RECENT ADVANCES IN BIOCHEMISTRY

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RECENT ADVANCES IN BIOCHEMISTRY

BY

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SECOND EDITION

WITH 38 ILLUSTRATIONS

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TO

MY FRIEND

WILLIAM OGILVY KERMACK

M.A., D.Sc.

Printed in Great Britain.

PREFACE TO THE FIRST EDITION

Biochemistry is a science of such recent development and active progress that the whole of our subject might well be embraced by the title of this little volume. My chief problem has therefore been the choice of material. It has been my aim to include as diverse as possible a selection of topics, in which the recent advances have been most striking, and in which the trend of modern biochemical research is well indicated. One has but to contemplate the immense increase in biochemical literature since the war of 1914–18 to appreciate the magnitude of even this task. I realise, therefore, that to certain biochemists my selection of matter may seem somewhat arbitrary, but this is a defect inseparable from a work of this nature, in which the predilections of the author are bound to find expression. While therefore, in view of the limited space at my disposal, I make no apology for the inevitable omission of many aspects of biochemistry both interesting and instructive, there are two omissions which are in rather a different category. I refer to the mechanisms of tissue oxidations and the chemistry of the internal secretions. Both of these are fields of investigation in which the interests of biochemists are very active, and in which striking developments have recently occurred, while many more are in prospect. They have, however, been treated so adequately in Professor Lovatt Evans' volume "Recent Advances in Physiology," in the same series as the present volume, that I have judged it unnecessary and unwise to re-traverse these fields. Rather I have preferred to devote the space, which might have been utilised for this purpose, to certain aspects of two subjects which do not

usually find inclusion in text-books of biochemistry. These are the chapters on " The Chemical Basis of Specific Immunological Reactions " and on " Chemotherapy," subjects which have a special appeal to medical readers and a challenging interest to biochemists and chemists alike.

It is with great pleasure that I acknowledge my indebtedness to Professor J. C. Drummond, D.Sc., for reading and checking my manuscript of the chapter on vitamins, and to Dr. J. H. Shaxby and Dr. W. H. Tytler for similar services in regard to Chapters II. and X. respectively. I am also indebted to Dr. W. O. Kermack for much advice and criticism while the book was in manuscript form. My special thanks are due to my colleague, Mr. J. M. Peterson, B.Sc., for much painstaking labour in the preparation of drawings for the illustrations and for assistance in the preparation of the index. My thanks are likewise due to Professor Leathes, F.R.S., for the loan of the blocks and photographs from which figures 11, 12, 13, and 14 are reproduced ; to Messrs. Ogilvy & Co., 20, Mortimer Street, W. 1, for the loan of the block from which figure 25 was made ; and to Messrs. The McGraw-Hill Book Company, Inc., of New York, for permission to reproduce figures 2, 3, and 6 from Loeb's " Proteins and the Theory of Colloidal Behaviour." I am also indebted to Professor Lovatt Evans, F.R.S., and to the publishers, Messrs. J. & A. Churchill, for sending me a proof copy of " Recent Advances in Physiology," thus enabling me to plan my own book with the avoidance of undue repetition in fields of cognate interest to physiology and biochemistry. I feel, too, that a final word of gratitude is due to Messrs. Churchill, not only for their unfailing assistance and courtesy, but also for the admirable manner in which they have given expression to their art in the form of the present volume.

J. P.

Physiology Institute,
Newport Road, Cardiff.

PREFACE TO THE SECOND EDITION

The active progress of biochemistry referred to in the preface to the first edition of this book is well illustrated by the necessity for numerous alterations and additions which had arisen within some twelve months of the first appearance of the book. The exhaustion of the first edition has provided a suitable opportunity for a complete revision.

The chief additions which have been made are as follows. A new chapter, Chapter IV., has been added dealing with the important work of Harington on the chemistry of thyroxine, the specific principle of the thyroid gland, and with the advances made by Raper and his school in the investigation of the formation from tyrosine of the widespread animal pigment melanin under the action of the enzyme tyrosinase. Additions have been made to Chapter V.; thus the newer work of Haworth, Hirst and their co-workers establishes beyond all reasonable doubt the general structure of both normal and reactive sugars, and formulæ and text have been revised accordingly. Opportunity has also been taken to include the recent work on the *rôle* of insulin in carbohydrate utilisation. In Chapter VII., dealing with the biochemistry of phosphorus compounds, considerable additions have been necessary. The important work of Morgan and Robison on the structure of the hexose diphosphoric acid of yeast fermentation appeared too late for inclusion in the text, but by a happy chance the formula II. given on page 169 (page 147, 1st Edition) represents the structure which they are now able to assign to this interesting compound. The recent work of Emben and Zimmermann on lactacidogen and adenylic acid in muscle, of Meyerhof on the lactic acid-

forming enzymes of mammalian muscle, of Eggleton and of Fiske on the interesting creatine-phosphate compound of muscle, and of Channon and Chibnall of the new phosphatide, calcium phosphatidate, isolated from plant sources, all find a place in this chapter. In Chapter IX. the chief additions deal with the differentiation of water-soluble vitamin B into its two components which are referred to as vitamins B_1 and B_2 in accordance with a recent recommendation of the Accessory Food Factors Committee, and with the highly important advance, rendered possible by the work of Rosenheim and Webster and of Windaus, which suggests very strongly that the precursor of vitamin D, the anti-rachitic vitamin, is ergosterol, and that the latter is directly transformed into the vitamin under the influence of ultra-violet radiations. In Chapter X. the chief revisions have been necessitated by the more recent work on the nature of hæmoglobin by Hill and Holden, and a new scheme has been given on page 298 in place of that which formerly appeared on page 269. Fischer and Klarer's synthesis of ætioporphyrin has also been included in this chapter. The progress made by Heidelberger and Goebel in the study of the specific carbohydrates of *pneumococcus* and related organisms has been included in Chapter XII.

My thanks are due to Professor Raper, of Manchester University, for kindly placing at my disposal the manuscript of an article on the tyrosinase-tyrosine reaction which he had prepared for "Fermentforschung."

I am greatly indebted to Messrs. J. and A. Churchill for the ample facilities afforded by them for revising this book.

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But nature, who loves to veil herself at our approach, works her transformations in mysterious, hidden laboratories; and it being granted that the human body contains chalk, sulphur, phosphorus, iron and ten other substances besides, it is hard indeed to find the explanation of how all this can be sustained and renewed during a period of years by bread and water.

Brillat-Savarin (*Physiology of Taste*, 1825).

RECENT ADVANCES IN BIOCHEMISTRY

CHAPTER I

ASPECTS OF PROTEIN CATABOLISM. AMINO ACIDS AND UREA

The biochemical importance of the α-amino acids needs no emphasising. These acids are the ultimate products of the hydrolysis of proteins, and therefore represent the chief source of nitrogen available to the animal body. When not required for the synthesis of more complex bodies, they are readily oxidised in living tissues, their nitrogen being excreted in the form of urea, and to a less extent as ammonia. The relative extent and ease of occurrence of these processes vary with the amino acid. In the case of the essential amino acids which the animal cannot synthesise for itself, and which are required for the elaboration of specific proteins or other products of cellular activity, deaminisation and oxidation probably occur in the normal course of events to a much less extent than in the case of the simpler amino acids. Thus cystine, phenylalanine, and tyrosine are included in those amino acids which, when injected into the animal, do not immediately produce an increase in urea formation such as is observed to occur in the case of glycine and leucine. The nitrogen of the latter acids is excreted readily and completely in the form of urea. Traces of amino acids are normally to be found excreted in the urine, but in general the utilisation of most amino acids is practically complete.

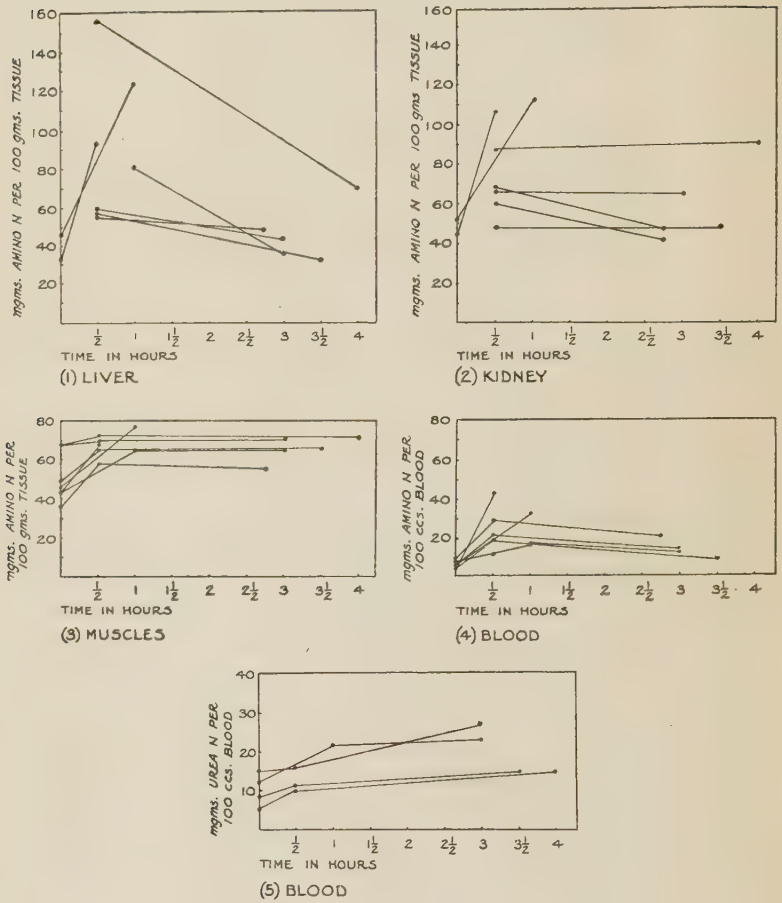

mgms. AMINO N PER 100 gms. TISSUE
TIME IN HOURS
(1) LIVER
mgms. AMINO N PER 100 gms TISSUE
TIME IN HOURS
(2) KIDNEY
mgms. AMINO N PER 100 gms. TISSUE
TIME IN HOURS
(3) MUSCLES
mgms. AMINO N PER 100 ccs. BLOOD
TIME IN HOURS
(4) BLOOD
mgms. UREA N PER 100 ccs. BLOOD
TIME IN HOURS
(5) BLOOD

FIG. 1.—Variations in amino nitrogen of liver, kidney, muscles, and blood, and in urea of blood after the injection of amino acids. Drawn from data of van Slyke and Meyer.

The Process of Deaminisation

The process by which the amino acids are utilised after they have been absorbed from the intestine into the blood stream has been studied in recent times by many schools of

biochemists, notably those of van Slyke and Folin. As a result of the injection of amino acids into the venous circulation, there is a rapid increase in the amino nitrogen of the liver, this organ taking up in the space of a few minutes some 150 to 160 mg. of amino nitrogen per 100 grams of fresh tissue. The amino nitrogen of the general blood circulation is likewise temporarily increased, and a large proportion of this increase is rapidly taken up by other organs, such as the kidney and muscles. In the case of the liver the maximal uptake of amino nitrogen is soon followed by a steady diminution, and at the same time the blood urea increases. The post-absorptive fall in amino nitrogen is not nearly so well marked in the case of the kidney and muscles. Some of the results of van Slyke are shown in the charts reproduced in Fig. 1. A point of interest which has emerged in these investigations is the great rapidity with which amino acids temporarily accumulating in the blood stream are transferred to the tissues.

The Mechanism of Deaminisation

During the absorption of amino acids from the intestine, the blood, in its passage through the liver, loses more amino nitrogen than it does in passing through all the other organs of the body. From these observations we conclude that the liver plays an important part in the general metabolism of the amino acids. As a result of their passage through this organ they undergo extensive deaminisation, and the liberated nitrogen is converted to urea. But it must not be assumed that this function of deaminisation and urea formation is purely hepatic. It may occur in animals in which the liver has been cut off from the general circulation, but in these cases it is well known that the amount of urea formed diminishes, whilst the ammonia is increased. We may, however, safely conclude that the liver is the chief seat of the functions which we are discussing. It has been assumed that the amino nitrogen is liberated in the form of ammonia,

and that the latter is then converted to urea by combination with carbon dioxide, passing through the intermediate stages of ammonium carbonate and carbamate. We shall return later to a discussion of the mechanism by means of which amino nitrogen is converted into urea, but meanwhile it may be noted that the direct formation of ammonia from amino acids is very doubtful. Folin and Denis found no increase in the ammonia of the portal blood after injecting the amino acids glycine and asparagine, and they have shown that much of the ammonia present in the portal blood originates from bacterial decomposition in the intestine.

Whatever the change may be by means of which the removal of the nitrogen from the amino acid molecule is effected, there is general agreement that it involves a process of α-oxidation. By α-oxidation we mean that the α-carbon atom of the original amino acid to which the amino group was attached has become oxidised during the process of deaminisation. O. Neubauer was the first investigator to bring forward experimental evidence in support of the view that this process involved the production of an α-ketonic acid. In 1883 Schotten had found that after administering phenylglycine (phenylaminoacetic acid) to dogs he recovered in the urine, in addition to some unchanged acid, the corresponding hydroxy acid, namely, mandelic acid. This latter acid must have originated from the phenylglycine given to the animal at the commencement of the experiment, and it would seem to have originated by a process of hydrolytic deaminisation, thus :—

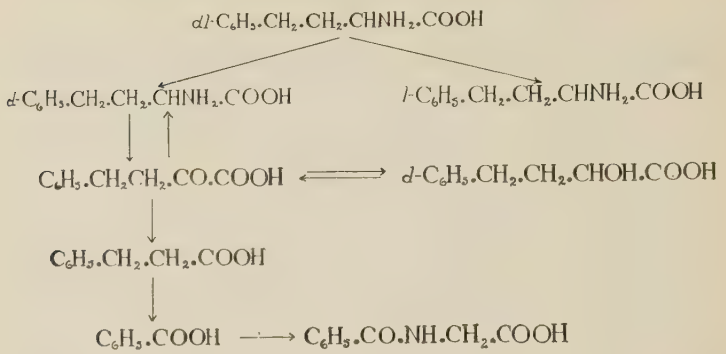
$$\underset{\text{(Phenylglycine.)}}{C_6H_5.CHNH_2.COOH} + H_2O \longrightarrow \underset{\text{(Mandelic acid.)}}{C_6H_5.CHOH.COOH} + NH_3.$$

That this was not the case was shown in Neubauer's investigations on the fate of optically inactive phenyl α-aminoacetic acid in the dog, and in the isolated perfused liver of the dog. In both instances he found the following products : (1) unchanged phenylaminoacetic acid containing excess of the

lævo-rotatory compound ; (2) *lævo*-mandelic acid ; (3) phenylglyoxylic acid, $C_6H_5 . CO . COOH$; (4) benzoic acid. The last-mentioned acid is found in the urine as hippuric acid, $C_6H_5 . CO . NH . CH_2 . COOH$. On administering the *dextro*-rotatory isomeride of phenylaminoacetic acid to the dog he also found in the urine phenylglyoxylic acid and *lævo*-mandelic acid. It seems evident from these experiments that the *dextro*-rotatory acid is more readily metabolised than the *lævo* acid, that the ketonic acid is the main product of the deaminisation, and that the mandelic acid must arise by an asymmetric reduction of the inactive ketonic acid so formed, since *lævo*-mandelic acid could not be formed directly from the *dextro*-amino acid. The series of changes may be represented as follows :—

$$C_6H_5 . CHNH_2 . COOH \longrightarrow C_6H_5 . CO . COOH \longrightarrow C_6H_5 . CHOH . COOH$$
$$\downarrow$$
$$C_6H_5 . COOH.$$

Many other instances of a similar series of changes have since been brought to light. Special importance attaches to Knoop's investigations. In these γ-phenyl α-aminobutyric acid was administered to dogs in the form of the optically inactive acid. There were recovered in the urine the *lævo* and *dextro* isomers of the unchanged acid, *dextro*-phenyl α-hydroxy-butyric acid, the corresponding α-ketonic acid, and hippuric acid. In this case, again, the hydroxy acid was proved to have originated from the ketonic acid by a process of asymmetric reduction, since direct administration of the sodium salt of the ketonic acid led to the excretion of the *dextro*-hydroxy acid. Knoop made a further very important discovery in this last stage of the experiment. He also found in the urine the acetyl derivative of *dextro*-phenyl α-amino-butyric acid, proving conclusively that the ketonic acid had been utilised in synthesising this amino acid. Dakin has represented this important series of investigations in the following schematic way :—



It will be seen that the stage represented above, in which phenylpropionic acid, resulting from the oxidation of phenyl α-ketobutyric acid, is converted to benzoic acid, involves the removal of two carbon atoms. This process, which we shall discuss in Chapter VI., is known as β-oxidation. Dakin summarises the results of these and many other experiments on the fate of amino acids in the animal body in the following terms : " In general the fate in the body of α-amino and α-ketonic acids is identical, whereas the α-hydroxy acids being presumably secondary reduction products may behave differently. It is therefore assumed that α-ketonic acids are obligate products of the direct oxidation of amino acids, while the hydroxy acids are not directly derived from the amino acids. A ketonic acid may therefore undergo three types of change : (1) it may be oxidised to a lower fatty acid ; (2) it may be reduced to a hydroxy acid ; (3) its ammonium salt may be reduced to an amino acid."

Knoop's demonstration of the synthesis of an amino acid from the corresponding ketonic acid is of fundamental importance, and soon Embden and his school showed that the reaction is a general one. They obtained alanine, phenylalanine, and tyrosine on perfusing the ammonium salts of the corresponding ketonic acids through the isolated liver. In the experiments of Neubauer and of Knoop there is no

doubt that the hydroxy acids were secondary products arising from the reduction of the ketonic acids, but the reverse change of hydroxy acid to ketonic acid is one which the tissues are also capable of effecting. Thus not only did Embden find that pyruvic acid yielded alanine on being perfused through the liver, but that the corresponding hydroxy acid, lactic acid, likewise gave rise to the amino acid. We have, therefore, as a normal feature of metabolism the closest correlation between these three types of acids, and their interconversion is strictly reversible, thus :—

$$CH_3 . CHNH_2 . COOH \rightleftarrows CH_3 . CO . COOH \rightleftarrows CH_3 . CHOH . COOH$$

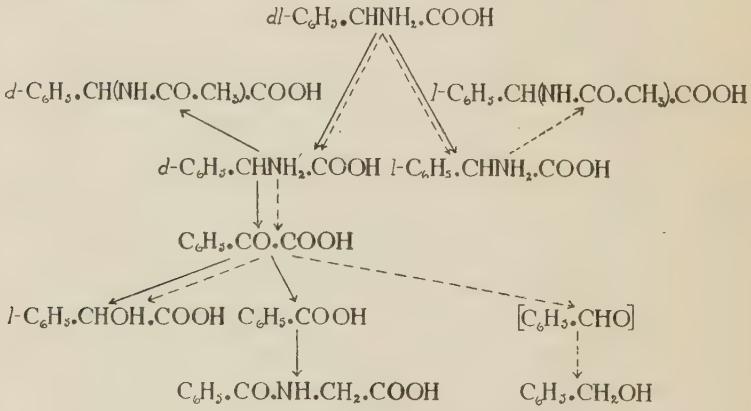
As we shall see in a later chapter, both lactic and pyruvic acids are of the greatest importance in carbohydrate metabolism, and these observations provide a sound theoretical basis for the well-known conversion of carbohydrate into amino acids, and ultimately into protein, and also for the reverse change, which is readily demonstrated in the diabetic subject and in the phlorhizinised animal.

The type of biochemical reaction which we have discussed in the foregoing paragraphs, namely, deaminisation accompanied by production of the α-ketonic acid, is by no means confined to the mammalian organism, or even to the animal kingdom. Neubauer included in his investigations a study of the action of yeast on *dl*-phenylaminoacetic acid, and found that a series of compounds very similar to those produced in the dog could be isolated as a result of the activity of this simple organism. From the culture medium containing yeast and the amino acid the following products were isolated : (1) unchanged *l*-phenylaminoacetic acid and its acetyl derivative, phenylglyoxylic acid, *l*-mandelic acid, and benzyl alcohol. It was also shown that, as in the animal, the *l*-mandelic acid arose from phenylglyoxylic acid by reduction, and that the reverse change could also take place but much less vigorously than the former change. The importance of the ketonic acid as a precursor of the benzyl

alcohol, which in this instance represented the end product of the series of reactions, was shown by the conversion in a similar experiment with yeast of another ketonic acid, *p*-hydroxyphenylpyruvic acid, into the corresponding alcohol with one carbon atom less, *p*-hydroxyphenylethyl alcohol :

$$HO.C_6H_4.CH_2.CO.COOH \longrightarrow HO.C_6H_4.CH_2.CH_2OH.$$

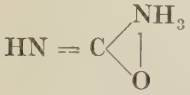
As we shall see in a later chapter (p. 124), such a change probably involves the formation of the aldehyde, in this case benzaldehyde, as an intermediate step. The essential similarity of the two series of transformations in which phenylaminoacetic acid figures in the dog and in yeast cultures is shown in the following scheme. The continuous line represents changes taking place in the mammal, whilst the broken line represents those occurring in yeast :



In both cases it should be observed that the *dextro* isomer is preferentially metabolised, and that it is the *lævo* form of mandelic acid which is formed by asymmetric reduction of the ketonic acid. On the other hand, an interesting difference between the two processes is the production of the *dextro* form of phenylacetylaminoacetic acid in the dog and of its optical antipode, the *lævo* form, in the yeast fermentation.

The Structure of Urea

Before passing on to a discussion of the mechanism of urea formation, we must first consider the structure of this compound. In recent years our ideas concerning the structure of this fundamental product of amino acid catabolism have undergone a considerable change. In 1913 E. A. Werner suggested that urea in its stable form had the cyclic structure shown below instead of the familiar carbamide structure usually attributed to it :—



(Urea—Cyclic Formula.)

(Carbamide.)

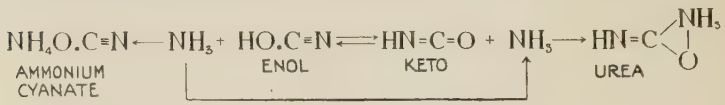
This suggestion is of great biological significance, and we shall first briefly review the chemical evidence which has been discussed and amplified by Werner in support of his views.

These views are based very largely on a reinvestigation of the close relationship which exists between urea and its derivatives on the one hand and cyanic acid on the other. This relationship has been known ever since Wöhler's classical synthesis of urea, which, it will be remembered, involved the intramolecular rearrangement of ammonium cyanate. This change, which proceeds, be it noted, as a bimolecular reaction, may be represented as follows :—

$$NH_4O \,.\, C \equiv N \text{ or } NH_4 \,.\, N = C = O \longrightarrow O = C = (NH_2)_2.$$

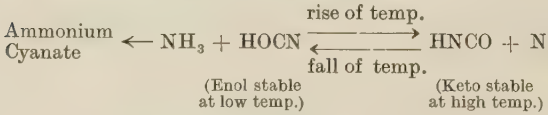
The change takes place even at ordinary temperatures on exposing ammonium cyanate to the air, and is complete after two days. While the conversion is proceeding ammonia is being constantly given off. On the other hand, if the ammonium cyanate be enclosed in an atmosphere of ammonia, there is no conversion to urea even after it has remained at room temperature for eight days. Werner's explanation of these observations is that ammonium cyanate

dissociates, like most ammonium salts, with considerable ease. It thus gives rise to ammonia and cyanic acid. The latter is liberated in the enolic form, and in an effort to attain a more stable configuration it is transformed into the keto form, *iso*cyanic acid. The *iso*cyanic acid then recombines with ammonia to form urea. The change is simply represented in the following way :—



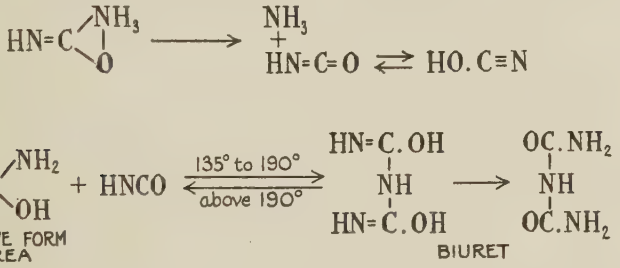
This view of the mechanism of the conversion of ammonium cyanate into urea is based essentially on the supposition that cyanic acid is comprised of two different molecular forms in equilibrium. It ought, therefore, to be possible to devise conditions which lead to the reversal of the above transformation. This is exemplified in the partial reconversion of urea to ammonium cyanate which takes place in aqueous or alcoholic solutions at a temperature much below that at which urea in bulk is known to change.

On being subjected to heat urea exhibits a series of changes the mechanism of which have an important bearing on its structure. These changes are correlated by Werner with the equilibrium system, which may be represented as follows :—

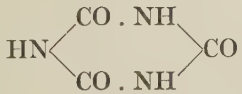


If urea is heated in a vacuum at 160° a sublimate is formed which is found to consist of ammonium cyanate. The same sublimate is also formed when urea is heated in the ordinary way, and further heating leads to the familiar formation of biuret, which is accompanied by the evolution of ammonia. When the heating is continued above 190° cyanuric acid and ammelide are formed. The first stage of these changes is the dissociation of the urea into ammonia and cyanic acid and

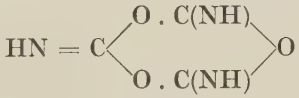
the recombination of some of these constituents in the cooler parts of the apparatus to form ammonium cyanate. The bulk of the cyanic acid formed in this high-temperature dissociation will be present in the keto form, and this on further heating combines with the reactive form of urea, which Werner figures as an open chain compound, to give the enolic form of biuret. The latter then changes to the more stable keto form. The formation of ammelide represents a further condensation of *iso*cyanic acid with biuret. These changes may be represented in the following way :—



Biuret, on being heated to still higher temperatures, decomposes to form ammonia and cyanic acid, which volatilise, considerable quantities of urea and cyanuric acid, and small quantities of ammelide. Cyanuric acid is also formed by polymerisation of cyanic acid at high temperatures. It has the constitution shown below. The polymerisation of cyanic acid at low temperatures leads to the formation of cyamelide.



(Cyanuric Acid.)



(Cyamelide (Hantzsch's Formula).)

Werner has explained how the formation of these two polymers of cyanic acid may be correlated with the stability of the ketonic and enolic forms of the parent acid at high and low temperatures respectively. It will be seen from the above equations that the formation of biuret is a reversible reaction, since at temperatures above 190° it is dissociated with the formation, amongst other products, of the compounds from which it originated, namely, cyanic acid and urea. Many years ago Finckh demonstrated the formation of biuret by passing the vapour of cyanic acid into urea at its melting point (132°). Werner has described a method for demonstrating a similar formation of biuret in aqueous solution at low temperature.

To 2 grams of urea dissolved in 2 c.c. of strong hydrochloric acid, diluted with 4 c.c. of water, 2 grams of powdered potassium cyanate are gradually added with constant stirring; after a few moments the solution is filtered to separate some cyamelide which has been formed. The filtrate, when tested in the usual manner with copper sulphate and excess of alkali, will be found to give a strong biuret reaction.

Further evidence in favour of the new mode of formulating urea is forthcoming from a consideration of the interaction of urea and nitrous acid. One might expect that if the carbamide formula correctly represented urea the reaction with nitrous acid would be a simple decomposition entirely analogous to the well-known mode of decomposition exhibited by substances containing an amino group, thus :—

$$OC(NH_2) + 2\ HNO_2 \longrightarrow CO_2 + 2\ N_2 + 3\ H_2O,$$

and the reaction ought to be available for the quantitative determination of urea. Such is not the case, and Werner, in investigating the behaviour of urea towards nitrous acid, has established the following points : (1) urea and pure nitrous acid do not interact in aqueous solution, but the presence of a mineral acid quickly initiates a brisk reaction ; (2) a

weak acid, such as acetic, does not promote the interaction unless the concentration of the acid is very high, and even then the velocity of the reaction is very slow ; (3) the volume of nitrogen evolved does not correspond to that calculated on the basis of the equation given above, the quantity of urea decomposed being much greater than that indicated by the evolved nitrogen ; (4) only when the urea is present in considerable excess is the volume of nitrogen obtained an approximate estimate of the amount of nitrous acid decomposed ; (5) the ratio of carbon dioxide to nitrogen in the gas evolved is always higher than that indicated in the equation. The absence of action of pure nitrous acid on urea indicates that an amino group, as such, is not present. It is interesting to note that thiourea, which according to Werner may exist in solution as $HN = C\begin{matrix}NH_2\\ \diagdown\\ \diagup\\ SH\end{matrix}$, is rapidly decomposed by pure nitrous acid, giving rise to thiocyanic acid, nitrogen, and water. When, however, in the case of urea a strong acid such as hydrochloric is present, a salt is formed which reacts according to the equations :—

$$HN = C\begin{matrix}NH_3\\ |\\ O\end{matrix} + HCl \longrightarrow HN = C\begin{matrix}NH_2 . HCl\\ \diagdown\\ \diagup\\ OH\end{matrix}$$

followed by

$$HN = C\begin{matrix}NH_2 . HCl\\ \diagdown\\ \diagup\\ OH\end{matrix} + HNO_2 \rightarrow N_2 + HNCO + 2H_2O + HCl.$$

The cyanic acid formed according to the second equation is then decomposed in two ways : (1) by hydrolysis ; (2) by interaction with nitrous acid, as follows :—

(1) $HNCO + H_2O + HCl \longrightarrow NH_4Cl + CO_2$;

(2) $HNCO + HNO_2 \longrightarrow CO_2 + N_2 + H_2O$.

The formation of cyanic acid during the course of these reactions can be demonstrated by its precipitation as the silver salt on adding silver nitrate. Werner has confirmed the above explanation of the course of the interaction between urea and nitrous acid by quantitative measurements.

The Hydrolysis of Urea

Urea may undergo hydrolysis in aqueous solution with or without the addition of acids or alkalis. Fawsitt first demonstrated that the hydrolysis of urea by water alone at 100° involved the formation of cyanate, and the presence of the cyanate radicle is easily shown by heating a solution of urea at the boiling point for a few minutes, cooling, and adding silver nitrate. In the presence of acids dissociation into ammonia and cyanic acid is likewise the first phase of the hydrolysis, the course of the whole reaction being represented in the following equations, proposed by Werner :—

Phase I.

$$HN=C\begin{matrix}NH_3\\|\\O\end{matrix} + HCl \longrightarrow NH_4Cl + (HNCO \rightleftharpoons HOCN).$$

Phase II.

$$(HNCO \rightleftharpoons HOCN) + H_2O + HCl \longrightarrow NH_4Cl + CO_2.$$

Since cyanic acid is hydrolysed very rapidly, the velocity of the whole reaction will be determined by the rate of dissociation of the urea, both products of this dissociation being removed practically as rapidly as they are formed. In the presence of alkali the hydrolysis may be pictured as occurring also in two phases, as follows :—

Phase I.

$$HN=C\begin{matrix}NH_3\\|\\O\end{matrix} + NaOH \longrightarrow NH_3 + NaOCN + H_2O.$$

Phase II.

$$NaOCN + 2\,H_2O \qquad NH_3 + NaHCO_3.$$

The Enzymic Hydrolysis of Urea

The ammoniacal or alkaline fermentation of urine is a change which has been investigated by biochemists for more than a century. Fourcroy and Vauquelin in 1798-9 showed that the ammonia formed during this fermentation process had its origin in the urea. In 1864 van Tieghem isolated the organism, which has since been called *Micrococcus ureæ*, responsible for the decomposition of the urea. Many other organisms are also capable of decomposing urea in the same way as *Micrococcus ureæ*, and in 1890 Miquel proposed the use of the name *urease* to indicate the enzyme common to these organisms which brought about the hydrolysis of urea to ammonium carbonate. In 1909 Takeuchi found urease in the aqueous extract of crushed soya beans, which are the seeds of the plant *Glycine hispida*. The enzyme has since been found in other leguminous plants, and is said to be present in the sword bean (*Canavalia gladiata*) and in the jack bean (*C. ensiformis*) in even larger amounts than in the soya bean. The presence of urease in many other plant sources has also been recorded. Urease has also been detected in small amounts in the liver of the horse and the dog. Its occurrence in this animal source is not regarded as having any physiological significance, and it is suggested that it is derived from ingested vegetable matter (Steppuhn and Utkin-Ljubovzov).

The enzyme urease appears to be remarkably specific in its action, since it will not promote the hydrolysis of the following substituted ureas at temperatures between 45° and 50°: methyl, ethyl, *sym*-dimethyl, *sym*-diethyl, *asym*-dimethyl, *n*-propyl, phenyl, piperidyl, nor of thiourea, urethane, and biuret. It is stated that pure *n*-butyl urea is decomposed by urease at 45° to 50°, and it is suggested that this is due to the relative instability of this urea as compared, for instance, with ethyl urea, leading to dissociation at a lower temperature. The optimum temperature for the action of urease has been placed at the remarkably high figure of

60°, and this may account for the fact that even ethyl urea is slowly attacked by the enzyme at 70°, which must be just below the temperature limit of its activity. Werner suggests that it is probable that if the enzyme does not promote the dissociation of a substituted urea below 80°, it cannot hydrolyse that urea in the ordinary sense. The specificity of urease would, therefore, seem to be largely determined by the temperature at which the urea forming the substrate begins to be dissociated.

It is generally agreed that before urease hydrolyses its substrate the latter is adsorbed by the enzyme. Some such theory is necessitated by Bayliss' observation that urease still produces an appreciable hydrolysis of urea in solutions containing 80 to 90 per cent. of alcohol, in which media the enzyme is quite insoluble. Following the adsorption phase, it has been assumed that the conversion of urea to ammonium carbonate involved the simple addition of water, with the possible intermediate formation of ammonium carbamate, thus :—

$$CO : (NH_2) \longrightarrow CO : (NH_2)(ONH_4) \longrightarrow CO : (ONH_4)_2.$$

It seems natural that such a view should be held so long as urea is regarded as a diamino compound such as carbamide. But, as we have seen, there are very good grounds for formulating urea in an entirely different manner, and Fearon has recently investigated the mechanism of the action of urease from the standpoint of the cyclic formula proposed by Werner. He comes to the conclusion that the course of the reaction is not that outlined above. Fearon has shown that it is possible to isolate cyanic acid in the form of its silver salt by adding silver nitrate to solutions of urea undergoing decomposition by urease. He was able to obtain no evidence of the formation of ammonium carbamate during this process. He therefore formulates the theory that ammonia and cyanic acid are intermediate products of the action of the enzyme on urea, these products arising by the simple process of

dissociation which we have already discussed. Urease is, according to this view, a dissociating enzyme. Following the stage of dissociation the cyanic acid liberated is hydrolysed by the water present, and gives rise to ammonia and carbon dioxide. The latter then combine to form ammonium hydrogen carbonate, which is converted to the normal carbonate by a further combination with the ammonia liberated during the first or dissociation stage, which ammonia remains adsorbed by the enzyme until it is removed to form ammonium carbonate. The enzyme is, therefore, free to combine with more urea, and the cycle may begin afresh. Fearon adduces experimental evidence in support of the view that urease can adsorb both urea and ammonia.

The cyclic structure of urea has been accepted by Mack and Villars in their studies of the same reaction, but these workers still attach importance to ammonium carbamate and carbonate as intermediate products in the enzymic decomposition of urea. Mack and Villars, and also Kay, have been able to demonstrate a slight amount of urea formation by acting on strong solutions of ammonium carbamate and carbonate with urease. This it is claimed establishes the reversible nature of the action of urease, a claim which is probably justified. But it would seem to be in opposition to the views which we have just discussed. The last-mentioned authors suggest that the reaction which is catalysed by the enzyme is the conversion

$$\text{Urea} \rightleftharpoons \text{Ammonium carbamate.}$$

Mack and Villars admit that there is simultaneous formation of ammonium cyanate along with the production of carbamate, but they regard the production of the former compound as due to the spontaneous dissociation of the urea uninfluenced by the enzyme. As we have stated, however, Fearon has failed to detect any formation of ammonium carbamate during the action of urease on urea, and he argues that the product obtained by Mack and Villars

consists of cyanate only. Moreover, in the absence of the enzyme, cyanate is not formed spontaneously from urea under conditions leading to its formation when the enzyme is present. It is therefore difficult to believe that the presence of the enzyme is not definitely related with the appearance of cyanate. A further point of importance which is emphasised by Fearon is that urease does not catalyse the rate of formation of *ammonium cyanate* from urea, but that its action consists in the liberation of the unstable form of cyanic acid, in this case the keto acid. The latter is then hydrolysed until the progress of the reaction results in the accumulation of sufficient ammonia to retard the action of the enzyme through the formation of an adsorption compound to the exclusion of the substrate, and to retard the hydrolysis of the keto-cyanic acid. The argument that urease does not catalyse the reverse reaction of the conversion of ammonium cyanate into urea, which is used by both Mack and Villars and by Kay, is therefore not regarded by Fearon as valid. Granted that such is the case, the production of urea by the action of urease on mixtures of ammonium carbamate and carbonate still awaits an explanation. It should, however, be noted that only very small amounts of urea have been obtained by this process, and it seems possible that such amounts might be produced through the intermediate formation of cyanic acid. Indeed, Werner has demonstrated that the formation of urea by the action of heat on ammonium carbamate does involve this intermediate step.

Urea Formation in Living Tissues

It is obvious that the foregoing observations have an important bearing on the mechanism of the formation of urea as a sequel to the deaminisation of amino acids which occurs in living tissues, and we must now turn to a consideration of this question. We have already seen that the first step in the catabolism of amino acids consists of a process of α-oxidation and simultaneous deaminisation, and that the

amino group so removed is eventually excreted as urea. This process has generally been represented in the following way :—

$$\mathrm{R.CHNH_2.COOH} \begin{cases} \nearrow \mathrm{R.CO.COOH} \\ \searrow \mathrm{NH_3} \end{cases} \longrightarrow \mathrm{(NH_4)_2CO_3} \longrightarrow$$
$$\longrightarrow \mathrm{(NH_4O).CO.NH_2} \longrightarrow \mathrm{CO(NH_2)_2}.$$

There is no doubt that ammonium salts, such as the carbonate and chloride, either in perfusion experiments or when administered directly to animals, lead to an increased production of urea. This observation would seem to support the supposition that urea formation takes place from free ammonia or from an ammonium salt. From time to time various objections have been raised to this view, and the possibility of deaminisation occurring without the direct liberation of free ammonia or the direct formation of an ammonium salt has been considered. There are many difficulties that may be urged against the view that free ammonia is formed even temporarily by the oxidative deaminisation of amino acids, and it is certainly difficult to see how this can occur without disrupting the carbon chain. Knoop's suggestion of the intermediate formation of hydrated imino acids may be cited as an attempt to circumvent some of these difficulties :—

$$\mathrm{R.C} \begin{cases} \mathrm{OH} \\ \mathrm{COOH} \\ \mathrm{NH_2} \end{cases} \longrightarrow \mathrm{R.C} \begin{cases} \\ \mathrm{COOH.} \\ \mathrm{NH.} \end{cases}$$

One may here refer to the observations made by Fosse and his co-workers, which demonstrate the ease with which ammonia becomes oxidised in alkaline solutions in the presence of organic compounds. These observations seem to render improbable the liberation of free ammonia in an oxidative change such as we have seen deaminisation to be. On the other hand, when mixed solutions of ammonium salts

and glucose or other organic substances are subjected to oxidation, both cyanic acid and urea are frequently produced. Special importance attaches to the results which Fosse obtained when he subjected casein and amino acids, such as asparagine and glycine, to oxidation. In these instances the amount of urea formed was very much increased on heating the product from the oxidation with ammonium chloride. Cyanate was doubtless formed during the oxidation, but insufficient ammonia was present to enable it to form urea. This deficiency was remedied by the addition of ammonium chloride and urea was produced in quantity.

In a discussion of these and other observations Werner entirely rejects the idea that urea is formed from carbon dioxide and ammonia during the oxidation of nitrogenous compounds. Having in view the important part played by cyanic acid in the chemical transformations of urea, Fearon and Montgomery have recently investigated the possible relationship of cyanic acid formation to oxidative deaminisation. On oxidising glycine and alanine with hydrogen peroxide in alkaline solution the formation of cyanic acid and of urea was detected. Much more decisive results were obtained in the case of glycine on using potassium permanganate in an alkaline medium as the oxidising agent. Cyanic acid formation was specially marked when glucose or formaldehyde was present along with the amino acid. Reasons are advanced for regarding this cyanic acid as arising directly in the process of oxidative deaminisation. It is, of course, possible that it might be formed as a secondary oxidation product of ammonia liberated directly from the amino acid and subsequently oxidised to cyanic acid in the presence of other organic compounds, just as Fosse observed in his experiments. Cyanate was, however, detected during the mild oxidation of glycine, when the ammonia liberated was only 1·5 per cent. of the theoretical value for complete deaminisation. Moreover, in a solution of glycine slowly oxidised at 45° during a period of forty-eight hours the

increase in ammonia was not accompanied by a corresponding rise in the cyanic acid formed. This suggests that the ammonia is formed by the continuous hydrolysis of the cyanic acid at a rate which depends on the experimental conditions. Sarcosine (methyl glycine) gave greater yields of cyanic acid than glycine under similar conditions. These observations suggest to Fearon and Montgomery the possibility of a union between the amino group and an aldehydic or similar substance resulting from the transformation of other organic fragments undergoing oxidation. Subsequent oxidation of the amino acid following on this union would readily account for the production of the corresponding α-ketonic acid, thus :—

$$\begin{matrix} R.CH.NH_2 \\ | \\ COOH \end{matrix} + H.CHO \rightarrow \begin{matrix} R.CH.N:CH_2 \\ | \\ COOH \end{matrix} + 3O \rightarrow \begin{matrix} R.CO \\ | \\ COOH \end{matrix} + HNCO + H_2O.$$

How well fitted cyanic acid is to play the part of an intermediate substance in urea formation will be seen from the following considerations, advanced by Fearon and Montgomery : (1) in acid solution cyanic acid is completely hydrolysed to ammonia :—

$$HOCN + H_2O + HCl \longrightarrow NH_4Cl + CO_2;$$

(2) in neutral aqueous solution it is partly hydrolysed to ammonia and carbon dioxide, and partly converted to urea by combination with ammonia :—

$$HOCN \rightleftharpoons HNCO + H_2O \longrightarrow NH_3 + CO_2$$

$$HNCO + NH_3 \longrightarrow HN{=}C(NH_2)(O)$$

(3) in the presence of excess ammonia cyanic acid is completely converted into urea :—

$$NH_4OCN \rightleftharpoons NH_3 + HOCN \rightleftharpoons HNCO + NH_3 \longrightarrow HN{:}C(NH_2)(O)$$

The behaviour of cyanic acid in these respects is, therefore, in accord with the observed phenomena of acidosis, in which ammonia excretion is increased at the expense of urea formation. Cyanic acid, resistant as it is to further oxidation, is therefore well fitted to play an important part in the mechanisms which regulate the optimal hydrogen ion concentration of the tissues of the organism.

If these views are substantiated, it is clear that the simultaneous formation of the corresponding α-ketonic acid on the one hand, and cyanic acid on the other, necessitates a source of carbon other than the amino acid molecule which is itself being deaminised. Hence the significance of the increased yields of cyanic acid observed in the experiments described above when such an additional source of carbon was present in the form of glucose or formaldehyde. It may be that such a preliminary combination of the amino group with a carbon-containing fragment normally precedes the deaminisation *in vivo*. Cases in which such a type of combination has been known to occur are, therefore, highly significant. We have already seen (pp. 5, 8) that optically active acetyl derivatives of phenylaminoacetic and γ-phenyl α-aminobutyric acids have been detected in the products resulting from the deaminisation of these acids. Dakin has observed a similar formation of the acetyl derivative of p-methylphenylalanine in a metabolic investigation. One would not care to suggest that acetylation normally precedes deaminisation ; but may it not be a special aspect of a more general type of change ? It at least serves to indicate that living tissues can effect a substitution in the amino group similar to that required by the theory which we have just discussed.

The Significance of Urease in Plants

The occurrence of the enzyme urease in the vegetable sources which have already been mentioned raises the question of the presence of urea in the plant world, and of

the significance of the enzyme and its substrate in plant physiology. In the light of recent work urea can no longer be regarded as a product peculiar to animal metabolism. During the early part of the present century the presence of urea was detected in many fungi, and more recently it has been shown to be produced in many of the higher plants when these are allowed to germinate in media entirely free from urea. It is but natural, therefore, that urea and urease should be found in association with one another, and this has been shown to be the case in young green pea plants and in soya plants. The presence of urea in plants is particularly associated with the breakdown of proteins which occurs during germination of the seed. So frequent is this phenomenon that Werner suggests that urea is a constant product of the disintegration of protein in the plant world just as it is in the animal world. The breakdown of the stored protein of the seed may therefore be regarded as a phenomenon which involves the production of urea.

The plant, as we know, synthesises its protein from the simplest chemical compounds, ammonia or nitrates and carbon dioxide. In order that the materials of the disintegrated protein should become available for the building up of new cell proteins for the growing plant, the transformation of such urea as is formed into cyanic acid, and ultimately into ammonia and carbon dioxide by the agency of urease, seems a natural step. Werner suggests that the synthesis of new protein may commence at the cyanic acid stage and that the ultimate hydrolysis to ammonia and carbon dioxide, for which, in his view, the enzyme is not directly responsible, is not always necessary. We do know, however, that the plant does utilise these two simple substances, but it is, of course, possible that cyanic acid may be formed from them :—

$$CO_2 + NH_3 \longrightarrow HNCO + H_2O.$$

As a synthetic process this seems no more remarkable than

the well-known phytosynthesis of carbohydrate from carbon dioxide and water. We cannot as yet say what the next step in the process may be. Werner suggests that the cyanic acid formed from urea or from other nitrogenous compounds is combined with "nascent" carbohydrate. The whole group might then become attached to compounds of the type of pyruvic acid, a process more or less the reverse of that shown on p. **21**.

These considerations enable us to understand why urease is not found in the higher animals. The animal cannot utilise either ammonia or carbon dioxide; its deficient synthetic abilities render these simple compounds useless to it; they are definite end-products in the metabolic cycle, and there is therefore no reason for their formation from urea, itself an end-product.

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CHAPTER II

COLLOIDS AND THE PHYSICAL CHEMISTRY OF PROTEINS

When we call to mind the fact that living matter is comprised almost wholly of substances in a colloidal state, and that the bulk of these colloids are proteins, it is obvious that colloid chemistry, more especially that branch of colloid chemistry which has been built up round the proteins, must be intimately related to the more physical aspects of biochemistry. Graham in his earliest papers recognised this when he included albumin, gelatin, vegetable and animal extractive matters in his class of colloids.

In recent years it has become increasingly clear that the elucidation of those aspects of the colloidal reactions of proteins which are of importance to the biochemist is to be achieved from two main standpoints—firstly, by regarding the proteins, like the amino acids of which they are composed, as amphoteric electrolytes capable of participating in reactions in a strictly stoichiometric sense ; and secondly, by applying to their study the principles of Donnan's membrane equilibrium, since, although they are essentially electrolytes, their ions are large and non-diffusible. More than to any other individual worker, we owe to the late Jacques Loeb the foundation of these modern views of the colloidal behaviour of proteins on a rigid experimental basis.

The Behaviour of Proteins as Amphoteric Electrolytes

The amphoteric nature of the proteins—by which we mean their capacity to react either as bases or acids—is due to the presence of both amino and carboxyl groups, a fact which

may be expressed by the symbol $\left[\begin{matrix}P & -NH_2 \\ & -COOH\end{matrix}\right]$, P representing the remainder of the large protein molecule. It is clear that such a complex may form salts in two ways : (1) the basic amino group may combine with an acid such as hydrochloric acid to form a protein hydrochloride, or (2) the acidic carboxyl group may combine with a base such as sodium hydroxide to form a sodium proteinate. These two types of protein salts, in which the protein part of the compound forms the kation in (1) and the anion in (2), may undergo electrolytic dissociation or ionisation in the following ways, that part of the molecule which is capable of reacting with other substances being kept outside the brackets in this notation :—

(1) $$\left[\begin{matrix}P & -NH_2 \cdot HCl \\ & -COOH\end{matrix}\right] \rightleftharpoons \left[\begin{matrix}P & -\overset{+}{N}H_3 \\ & -COOH\end{matrix}\right] + \overset{-}{Cl}.$$

(2) $$\left[\begin{matrix}P & -NH_2 \\ & -COONa\end{matrix}\right] \rightleftharpoons \left[\begin{matrix}P & -NH_2 \\ & -COO^- \end{matrix}\right] + Na^+.$$

What are the conditions which determine the behaviour of a protein in one or the other of these two ways ? Loeb has shown that the hydrogen ion concentration of the solution in which the protein is present is the deciding factor.

Hydrogen ion concentration is usually expressed as the number of grams of ionic hydrogen (*i.e.*, hydrogen carrying a positive charge) in a litre of the solution under consideration at a stated temperature. In solutions of physiological importance this number is usually small, and for convenience it may be written as a negative power of the base 10 ; thus a concentration of 0·000001 gram of ionic hydrogen per litre may be written $[\overset{+}{H}] = 10^{-6}$. It is, however, customary in biochemistry to use the notation proposed by Sørensen, in which a particular ionic concentration is denoted by the

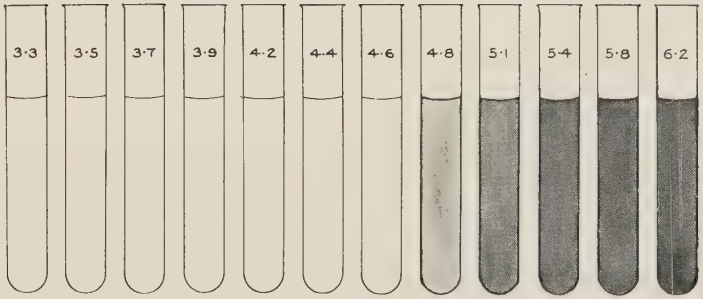

FIG. 2.—Proof that kations combine with proteins only on the alkaline side of the isoelectric point. The p_H is marked on each tube.
[After Loeb. By permission of Messrs. McGraw-Hill.]

[To face page 27.

symbol p_H, followed by the negative exponent only, omitting the negative sign. Thus a solution having $[\overset{+}{H}] = 10^{-6}$ is said to have $p_H = 6{\cdot}0$. Similarly a solution containing 0·000002 gram of ionic hydrogen per litre has $[\overset{+}{H}] = 2 \times 10^{-6}$. This is $10^{0{\cdot}3} \times 10^{-6}$ or $10^{-5{\cdot}7}$, so that $p_H = 5{\cdot}7$. Similarly $[\overset{+}{H}] = 4{\cdot}5 \times 10^{-8} = 10^{0{\cdot}65} \times 10^{-8} = 10^{-7{\cdot}35}$, so that $p_H = 7{\cdot}35$. For a further discussion of hydrogen ion concentration the reader is referred to Professor Lovatt Evans' book on "Recent Advances in Physiology."

The importance of the hydrogen ion concentration in controlling the behaviour of the proteins as amphoteric substances was illustrated by Loeb as follows. Portions of finely powdered gelatin each weighing 1 gram were brought to different hydrogen ion concentrations by immersing each for one hour at 15° in 100 c.c. of nitric acid solutions of concentrations varying from M/8 down to M/8192. The acid was then filtered off, and each portion of gelatin was washed once or twice at 5° with 25 c.c. of water to remove the greater part of the acid between the particles of gelatin. The portions of gelatin, which now each possessed a different p_H, were put for one hour into separate beakers, each containing M/64 silver nitrate at 15°. They were then removed and washed six or eight times with 25 c.c. of ice-cold water to remove excess silver nitrate not in combination with the gelatin. The latter was then melted by heating to 40°, and distilled water was added to each sample to bring the volume to 100 c.c. All the operations so far described were carried out in the dark. A portion of each solution was used to determine the p_H, whilst another portion of each solution was exposed in a test tube to strong light. The results obtained are shown in Fig. 2, the p_H of each sample being marked on the tubes. It will be seen that up to a p_H of 4·6 no darkening of the aqueous solutions has occurred, but above this p_H a sharp change is observed and all the solutions show darkening.

The inference drawn from this series of experiments is that at p_H greater than 4·7 (*i.e.*, lower hydrogen ion concentrations) the gelatin, which to a certain extent is present as sodium or calcium gelatinate, forms a silver gelatinate by double decomposition with the silver nitrate, *i.e.*, the protein ion behaves as an anion, thus $\begin{bmatrix} G - NH_2 \\ - COO^- \end{bmatrix} + Ag^+$. This silver protein salt is not readily washed out of the gelatin, and on exposure to light it exhibits the characteristic darkening shown by silver salts. At p_H less than 4·7 the gelatin formed the kation of a salt, in this case gelatin nitrate, $\begin{bmatrix} G - NH_3^+ \\ - COOH \end{bmatrix} + NO_3^-$, which could not react with silver nitrate. Hence all the silver nitrate was easily washed out and no darkening could occur on exposure to light. A similar series of experiments is illustrated in Fig. 3, in which the powdered gelatin, after being brought to different p_H, was treated for one hour with M/128 potassium ferrocyanide. After washing as before, the gelatin was melted and diluted to 100 c.c. After a few days the tubes in which the p_H was less than 4·7 turned blue, whilst those with higher p_H remained colourless. Hence at p_H less than 4·7 the gelatin forms the kation of a salt in which $Fe(CN)_6$ is the anion, and the latter betrays its presence by forming a blue ferric salt in the course of a few days. But above the p_H of 4·7 the gelatin is itself the anion, and therefore cannot combine with $Fe(CN)_6$, which is consequently washed out and no colour can develop.

We see, therefore, that in the case of gelatin the p_H 4·7 corresponds to a critical hydrogen ion concentration near which the protein shows a marked change in its behaviour. As we shall see later, many other properties of gelatin exhibit a characteristic change at this p_H, which is known as the isoelectric point of gelatin, since here it behaves neither as an acid nor as a base, or more strictly its ionisation in one

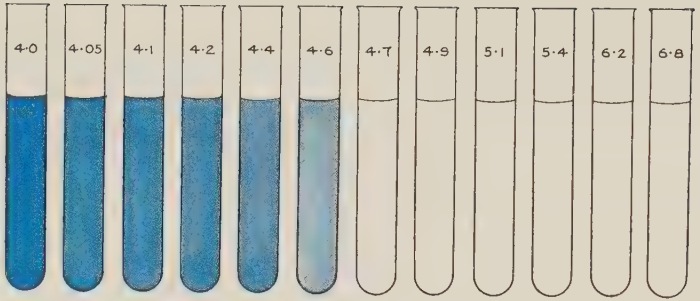

FIG. 3.—Proof that anions combine with proteins only on the acid side of the isoelectric point. The p_H is marked on each tube.

[After Loeb. By permission of Messrs. McGraw-Hill.]

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direction or in the other is minimal. It must, however, be pointed out that L. J. Harris has recently advanced arguments for regarding the isoelectric protein molecule not as a practically non-ionised molecule as we have supposed above, but as a molecule ionised *equally* as an acid and as a base, that is, as a type of internal ammonium salt. In this case the *total* ionisation (*i.e.*, both as acid and base) is *maximal* at the isoelectric point. At hydrogen ion concentrations above or below the isoelectric point we then have in place of what may be called the " combination " reactions shown on p. 26 the " replacement " reactions shown below :—

$$\underset{\text{(Protein Hydrochloride.)}}{\left[\begin{matrix} P\!-\!\overset{+}{N}H_3 \\ P\!-\!COOH \end{matrix}\right] Cl} \xleftarrow{HCl} \underset{\text{(Isoelectric Protein.)}}{\left[\begin{matrix} P\!-\!\overset{+}{N}H_3 \\ P\!-\!COO^- \end{matrix}\right]} \xrightarrow{NaOH} \underset{\text{(Sodium Proteinate.)}}{\left[\begin{matrix} P\!-\!NH_2 \\ P\!-\!COO^- \end{matrix}\right] Na^+} + H_2O.$$

Whether Loeb's original theory or the new one gives a correct representation of the protein molecule at its isoelectric point, both agree in regarding the latter point as being that p_H at which the protein is ionised equally as an acid and as a base, and consequently an essential part of both theories is the electrical neutrality of the molecule at the isoelectric point.

The position of this latter point was ascertained experimentally by Michaelis previous to the investigations of Loeb just cited. Michaelis showed that in cataphoresis experiments gelatin particles did not migrate to one pole or to the other when the p_H was 4·7, that is, they were electrically neutral, whereas at p_H either above this point or below it they were definitely charged and migrated, to the anode in the former case and to the kathode in the latter. This method of observing the hydrogen ion concentration at which protein particles do not migrate in an electrical field constitutes one means of determining the isoelectric point. We shall see that other methods which may be used for the same purpose are based on the fact that certain physical properties of the proteins, such as viscosity and osmotic pressure, are

minimal at the isoelectric point. Michaelis and Nakashima have recently described a convenient method of determining this point for proteins, which is based on the observation that the point of optimum precipitation of a mixture of gum mastic and gelatin in the presence of an acetate buffer approaches the isoelectric point of the gelatin as its relative amount is increased. In practice, therefore, a series of tubes containing mastic sol and an acetate buffer is made up for each p_H which is to be investigated. Increasing amounts of the protein whose isoelectric point is to be determined are added to each series of tubes. The p_H of the zone of optimum precipitation rises to a constant value beyond which further addition of the protein produces no further shift of the zone. This maximum constant value is the p_H of the desired isoelectric point.

The following are the isoelectric points of some common proteins :—

	p_H		p_H
Glutenin	4·45	Egg albumin	4·8
Gelatin	4·7	Serum globulin	5·4
Casein	4·7	Edestin	5·7
Serum albumin	4·7	Oxyhæmoglobin	6·75

The isoelectric point of a protein or similar colloidal amphoteric substance is of the greatest importance in relation to the biophysics of such compounds. We have stated that many of the properties of proteins exhibit a characteristic change at this point, and we have already seen that here the ionisation of the protein, either as an acid or as a base, is equal (see p. 29). Certain of these other characteristic properties which show a similar behaviour are the total swelling of the protein produced by the imbibition of water, the viscosity, the osmotic pressure, and, as one might expect, the conductivity. The "membrane potential," which we shall discuss later, also has a minimal value at the isoelectric point. The curves given in Fig. 4 illustrate these various phenomena, the experiments on which they are based being

described as follows by Loeb: "A number of doses, each containing 1 gram of finely powdered Cooper's gelatin which had a p_H of a little over 7·0 and consisted partly of calcium gelatinate, were put for thirty minutes at 15° into beakers containing 100 c.c. of HBr of different concentrations, varying from M/8 to M/8192; and, as a control, 1 gram of gelatin was put for thirty minutes at 15° into 100 c.c. of distilled water. The powdered gelatin was then put into a cylindrical funnel and the acid allowed to drain off. The powdered gelatin in the funnel was then perfused six or eight times with constant stirring, each time with 25 c.c. of cold water—*i.e.*, water not above 5° —to remove the excess of acid and the salts. The water must be cold to prevent the powdered granules from coalescing, since otherwise the washing would be incomplete. After the liquid was drained off from the filter, the volume

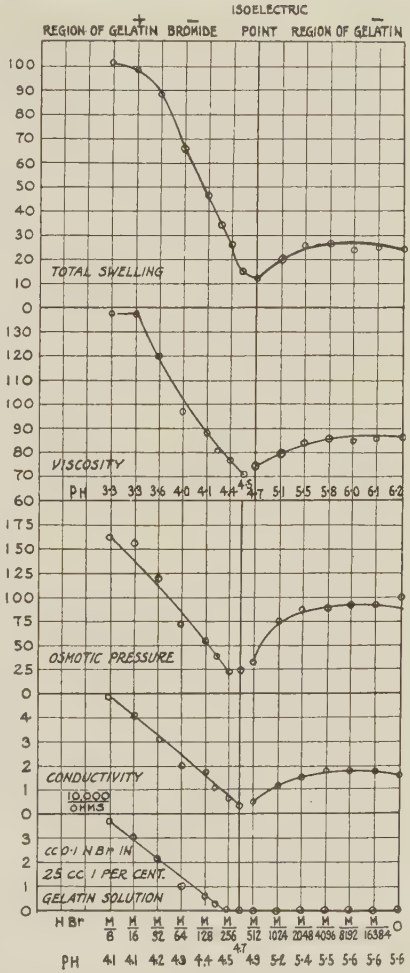

FIG. 4.—Curves showing that the total swelling, viscosity, osmotic pressure, and conductivity of gelatin are minimal at the isoelectric point, p_H 4·7. (After Loeb.)

(*i.e.*, the relative swelling of the gelatin) was measured; then the gelatin was melted by heating to 45°, and enough water was added to bring the volume in each case to 100 c.c. Then the conductivity, osmotic pressure, and viscosity were measured . . . and the p_H was also determined, either colorimetrically (which gives fairly accurate results with gelatin, but not with the other proteins) or, preferably, with the hydrogen electrode. In the experiment represented in Fig. 4 the p_H was measured colorimetrically. A glance at the figure shows that the ordinates of the curves representing the values for osmotic pressure, conductivity, swelling, etc., drop very sharply at p_H 4·7, *i.e.*, the isoelectric point of gelatin. By this method the approximate location of the isoelectric point can be recognised at a glance from the osmotic pressure measurements, the conductivity measurements, etc. The lowest curve in Fig. 4 represents titration for Br. Gelatin should exist in the form of gelatin bromide only on the acid side of the isoelectric point, and titration for Br should be negative when the p_H is above 4·7. The curve shows no Br was found when the p_H was equal to or greater than 4·7; while it was found on the acid side increasing in quantity the lower the p_H. On the alkaline side of the isoelectric point the gelatin existed still in the state of calcium gelatinate. In this experiment the mass of the gelatin was diminished by solution and washing to 0·8 gram, or possibly a little less."

Yet another property of the proteins which is minimal at the isoelectric point is their stability as emulsoid colloids, That is, their tendency to coagulate or precipitate is greatest at the point of electrical neutrality. This is probably because the attractive force between the dispersed protein and the surrounding water molecules is minimal at this point. Thus if 1 per cent. isoelectric gelatin in water be allowed to stand, and especially on cooling, a cloudy suspension will form and eventually precipitate, but no such separation of gelatin

occurs if the p_H is even slightly above or below the isoelectric point. Similarly, the addition of a little alcohol to isoelectric gelatin produces rapid precipitation, but at a p_H below 4·4 or above 5·0 the protein may withstand the addition of a considerable excess of alcohol without precipitating, if the anion of the acid and the kation of the alkali present be monovalent. If these are divalent, the increased precipitating action of these ions will cause precipitation to occur even at p_H 4·4 or 5·0. The facility with which heat coagulation of a protein is effected at the isoelectric point must be familiar to most readers. Isoelectric egg albumin in aqueous solutions will remain clear for months in considerable concentrations if the temperature be kept at about 2°; but if the temperature is raised, precipitation of 1 per cent. isoelectric albumin (p_H 4·8) will occur at 60°. This temperature is, therefore, that at which heat coagulation occurs at the isoelectric point. On altering the p_H to 4·4 by adding hydrochloric acid the heat coagulation temperature rises to 80°, and at p_H 4·25 or lower coagulation is incomplete even at 95°, an opalescence only being obtained. These observations demonstrate that the tendency of a protein to separate from its solution under the influence of precipitants or coagulants is maximal at its isoelectric point.

Another consideration of great practical importance in relation to the isoelectric point of proteins follows from the fact that at this point of minimal ionisation the protein must be associated with the minimum quantity of inorganic matter, and Loeb and others have shown that the ash content of commercial gelatin and other proteins is rapidly and conveniently brought to a very low value by adjusting the hydrogen ion concentration of the protein to that of the isoelectric point, using dilute acetic acid, and then by washing with cold water. Dialysis of a protein at its isoelectric point against dilute acid of the same p_H is, therefore, an important method of purification.

Influence of Ions on the Behaviour of Proteins and the supposed Hofmeister Series

The swelling, osmotic pressure, and viscosity of protein solutions are markedly influenced by the presence of salts, and Hofmeister, who first investigated these phenomena, drew up a series of salt anions in the order in which they were supposed to influence the properties mentioned. Such a series may be illustrated by the following sequence of ions, which shows the order of their activities in diminishing the osmotic pressure of gelatin :—

$$Cl > SO_4 > NO_3 > Br > I > CNS.$$

Loeb has, however, pointed out that these earlier investigations did not take into consideration the varying effect of the salts in which these ions were present on the hydrogen ion concentration of the solution. He has shown that if care be taken to adjust the hydrogen ion concentration to a common value before the comparison be made, the numerous complex Hofmeister series may be replaced by a simple valency rule. It can, for instance, be shown that the acetates influence the swelling, osmotic pressure, and viscosity of protein solutions in the same way, in a quantitative sense, as the chlorides and nitrates, but not in the same way as the sulphates and salts containing other divalent ions. These considerations apply also to kations, and of the properties of ions in general only the valency and sign of charge influence the colloidal behaviour of a protein, provided no constitutional change in the latter is produced. The Hofmeister series are, therefore, meaningless.

The Stoichiometric Behaviour of Proteins

Recognition of the fact that the proteins combine with acids and bases according to simple stoichiometric relations followed from the proof of their amphoteric nature. The most convenient method of demonstrating that these com-

binations take place in simple molecular proportions is by a study of the titration curves of the proteins. These curves are obtained by making up solutions of equal concentrations

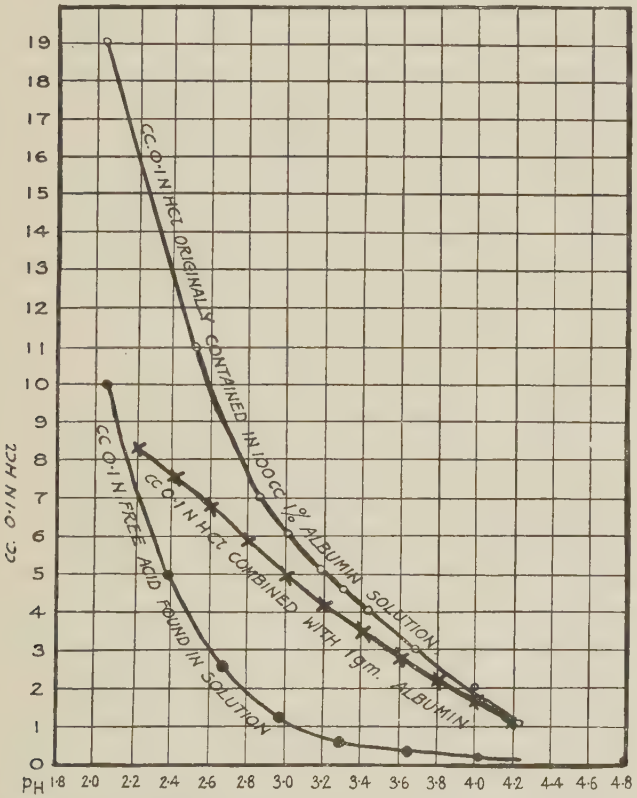

FIG. 5.—Titration curve and p_H curve of egg albumin showing method of determining the amount of acid in combination with 1 gram of albumin at any given p_H. (After Loeb.)

of the isoelectric protein containing varying concentrations of acids or alkalis, and then determining the p_H of the resulting solution, preferably with the hydrogen electrode. The titration curve is then obtained by plotting the con-

centration of acid or alkali against the p_H. From the titration curve one then obtains the amount of acid or alkali which is in combination with the protein at any given p_H by subtracting from the total amount added to bring the solution to that particular p_H, the amount of acid or alkali which is free. This latter amount is found by determining that concentration of acid or base which would be required to give the same p_H to an equal volume of pure water. Suppose that in 100 c.c. of originally isoelectric 1 per cent. egg albumin there is present 6 c.c. of n/10 HCl, and that the resulting p_H of the solution is found to be 3·0. To give this latter p_H to an equal volume (100 c.c.) of pure water one must add 1 c.c. of n/10 HCl, since the concentration of the resulting solution would be n/1,000 with respect to the acid, and assuming complete dissociation at this dilution, there would be present 0·001 gram of ionic hydrogen in a litre, *i.e.*, $[H^+]$ would be 10^{-3} or p_H 3·0. Therefore at p_H 3·0 5 c.c. of n/10 HCl are in combination with 1 gram of originally isoelectric egg albumin. In a similar way can be determined the amount of acid in combination with the egg albumin at any given p_H, and the curves given in Fig. 5 illustrate an actual series of experiments carried out by Loeb in this way.

On p. 37 are given the results obtained for the two proteins egg albumin and gelatin, using hydrochloric, sulphuric, oxalic and phosphoric acids, in four series of experiments on each protein.

In the case of both experiments detailed in these tables it will be seen that at any given p_H hydrochloric and sulphuric acids combine with the proteins in equivalent proportions, both acting as monobasic acids. Oxalic acid combines with the proteins in practically twice the amount of the hydrochloric or sulphuric acid at the same p_H, more especially below 3·2, whilst the amounts of phosphoric acid which combine with the proteins is, within experimental limits, three times that of the monobasic acids at the corresponding

TABLE I

Cubic Centimetres of n/10 Acid in combination with 1 gram of Originally Isoelectric Crystalline Egg Albumin in 100 c.c. of Solution (Loeb)

p_H.	HCl.	H_2SO_4.	Oxalic Acid.	H_3PO_4.
4·2	1·15	1·15	1·8	3·8
4·0	1·7	1·7	2·6	5·3
3·8	2·3	2·3	3·7	6·8
3·6	2·9	2·9	5·0	8·6
3·4	3·5	3·5	6·3	10·6
3·2	4·2	4·3	8·0	13·1
3·0	5·0	5·1	9·5	16·1
2·8	5·8	5·9	11·1	19·3
2·6	6·7	6·5	13·3	22·9
2·4	7·6	7·0	16·0	—

TABLE II

Cubic Centimetres of n/10 Acid in combination with 1 gram of Originally Isoelectric Gelatin in 100 c.c. of Solution (Loeb)

p_H.	HCl.	H_2SO_4.	Oxalic Acid.	H_3PO_4.
4·0	—	2·7	3·9	6·95
3·8	3·9	3·75	5·5	9·4
3·6	4·8	4·8	7·3	12·3
3·4	5·6	5·75	9·1	15·2
3·2	6·4	6·75	11·0	18·0
3·0	7·2	7·5	13·15	20·7
2·8	7·9	8·25	15·3	23·6
2·6	8·35	8·8	17·1	26·2
2·4	8·5	9·3	18·0	—

p_H. From the curve of combined HCl given in Fig. 5, and from the figures quoted above, it will be seen that a flattening tends to occur at about p_H 2. This would seem to indicate that the protein has a limited maximal capacity to combine

with acid, and that this maximum degree of combination is reached at the p_H quoted. These results are only to be explained on the basis of a simple stoichiometric combination between the protein and the acid, such as we encounter in the crystalloids. Interesting confirmation of such a view is forthcoming from a study of the titration curves of deaminised gelatin carried out by Hitchcock. It can be shown that the maximum combining capacity of the deaminised gelatin for hydrochloric acid is less than that of gelatin by an amount nearly equivalent to the free amino groups present in the original protein, and which can be shown to be removed by the process of deaminisation. The residual capacity for combining with acid shown by the deaminised gelatin must, therefore, be due to the peptide and other groups unaffected by the nitrous acid used in the deaminising process. Similar stoichiometric relationships are found to hold between alkalis and proteins at p_H above the isoelectric point. Thus in titration curves constructed for the five proteins egg albumin, gelatin, casein, serum globulin, and edestin, with the strong bases potassium, sodium, calcium, and barium hydroxides, the points obtained for any one protein with all of the bases fall on a single curve.

The acceptance of the view that proteins form compounds with acids and alkalis, first clearly established by the researches of Loeb and his associates, is now general. L. J. Harris, in a recent series of studies on the titration curves of amphoteric electrolytes, gives the following method for determining the combining or equivalent weights of proteins towards acids and alkalis. Increasing amounts of acid or alkali are added to a protein solution, and after each addition a measurement is made of a physical constant (either viscosity, total swelling, osmotic pressure, or conductivity, etc.). The latter is then plotted against the amount of acid or alkali added, not p_H. The curve is marked by a very sharp maximum or kink, which, in cases where the equivalent weight of the protein is already known from the combination curve

(or from conductivity), is found to coincide with the addition of exactly one equivalent of acid or alkali. The following results are from Harris' paper :—

TABLE III

Approximate Equivalent Weights of Proteins expressed in Terms of n/10 Acid or Alkali

Protein.	Method.	As a Base, c.c. n/10 Acid equivalent to 1 gram Protein.	As an Acid, c.c. n/10 Alkali equivalent to 1 gram Protein.
Gelatin . .	p_H-Combination curve	9	>6
	Swelling . . .	8·5	6·5–8·7
	Viscosity . .	9	—
	Specific conductivity	9	—
	Osmotic pressure .	9	—
Edestin . .	p_H-Combination curve	5·6	—
	Osmotic pressure .	5·6	—
Serum globulin	Osmotic pressure .	7	—
Casein . .	p_H-Combination curve	>7·5	>22
	Solubility . .	15	—
	Viscosity . .	15	—
	Swelling . . .	15	24
Egg albumin .	p_H-Combination curve	8·5	>9
	Osmotic pressure .	8·5 *ca.*	—

Harris concludes that a protein in solution may be determined volumetrically by titrating with standard acid or alkali between two given p_H points, the amount of titrant required being strictly proportional to the amount of protein present, and the estimation is as accurate as an ordinary acid-base titration. The practical application of such methods to the determination of the proteins of milk is illustrated as follows. Successive small amounts of n/10 hydrochloric acid

are run from a burette into a given volume of milk, and the p_H is determined after each addition. Three or four such readings are sufficient, as the result when p_H is plotted against cubic centimetres of acid added is practically a straight line, and readings at stated p_H values may be taken by intrapolation. The amount of acid required to take the milk from one given p_H value to another is directly proportional to the amount of protein present. Thus the number of cubic centimetres of n/10 hydrochloric acid required to titrate 10 c.c. of milk from p_H 6·65 to 5·2 is numerically equal to the percentage of protein in the milk. Readings should be taken only within the range of p_H 6·7 to 4. At values more acid than p_H 4 the blank correction begins to assume large magnitudes. By this method the percentage of protein in normal commercial cows' milk or modified milk mixtures may be determined to within 0·1 per cent. The blank correction mentioned represents the amount of acid required to take a hypothetical blank free from protein to the same final p_H value at the same final volume.

The Donnan Membrane Equilibrium and Proteins

If we imagine two solutions of electrolytes to be separated by a membrane which is permeable to all the ions save one present on one side of the membrane only, it can be shown that an unequal distribution of the diffusible ions must result. Thus if we start with a solution of NaR, R being a non-diffusible ion, on one side (1) of the membrane represented by the dividing line, and NaCl on the other (2), and consider the state of equilibrium attained by diffusion of NaCl from (2) to (1), we have first :—

Na^+	Na^+		Na^+	Na^+
R^-	Cl^-	and at equilibrium	R^-	
			Cl^-	Cl^-
(1)	(2)		(1)	(2)

In considering such a system Donnan states : " When this equilibrium is established the energy required to transport reversibly and isothermally 1 gram molecule $\overset{+}{\mathrm{Na}}$ from (2) to (1) equals the energy which can be gained by the corresponding reversible and isothermal transport of a gram molecule of $\overset{-}{\mathrm{Cl}}$. In other words, we consider the following infinitely small isothermal and reversible change of the system :—

$$\left.\begin{matrix}\delta n \text{ mol Na } (2) \longrightarrow (1)\\ \delta n \text{ mol Cl } (2) \longrightarrow (1)\end{matrix}\right\}$$

The energy which can be gained in this way (*i.e.*, the diminution of free energy) is zero, hence :—

$$\delta n \ \mathrm{RT} \log \frac{[\overset{+}{\mathrm{Na}}]_2}{[\overset{+}{\mathrm{Na}}]_1} + \delta n \ \mathrm{RT} \log \frac{[\overset{-}{\mathrm{Cl}}]_2}{[\overset{-}{\mathrm{Cl}}]_1} = 0$$

or

$$[\overset{+}{\mathrm{Na}}]_2 \cdot [\overset{-}{\mathrm{Cl}}]_2 = [\overset{+}{\mathrm{Na}}]_1 \cdot [\overset{-}{\mathrm{Cl}}]_1 \quad . \quad . \quad . \quad . \quad . \quad . \quad (\mathrm{A})$$

where the brackets signify molar concentrations." Thus the product of the concentrations of a pair of diffusible kations and anions must be equal on both sides of the membrane. But since on (1) $[\overset{+}{\mathrm{Na}}]$ is the sum of the kations in combination with both Cl and the non-diffusible ion R, and since on (2) $[\overset{+}{\mathrm{Na}}]$ represents only those kations combined with and equal to $[\overset{-}{\mathrm{Cl}}]$, it follows that $[\overset{+}{\mathrm{Na}}]_1 > [\overset{+}{\mathrm{Na}}]_2$ and $[\overset{-}{\mathrm{Cl}}]_1 < [\overset{-}{\mathrm{Cl}}]_2$.

Let us suppose that we have two systems, (A) gelatin chloride in equilibrium with hydrochloric acid, and (B) gelatin sulphate in equilibrium with sulphuric acid, in both of which the protein salt is separated from the acid by a membrane permeable to all the ions save that of the gelatin. The two systems may be represented as follows :—

$z\overset{+}{G}$	
$y\overset{+}{H}$	$x\overset{+}{H}$
$y + z\overset{-}{Cl}$	$x\overset{-}{Cl}$
(1)	(2)

A

$z\overset{+}{G}$	
$y\overset{+}{H}$	$x\overset{+}{H}$
$\frac{y+z}{2}\overset{-}{SO_4}$	$\frac{x}{2}\overset{-}{SO_4}$
(1)	(2)

B

Then using the symbols x, y, and z to denote the various ionic concentrations, or more strictly ionic activities, as indicated above, one can show thermodynamically that in system (A) $x^2 = y(y + z)$, and in system (B) $x^3 = y^2(y + z)$, assuming for purposes of simplicity complete dissociation of the gelatin salts and the acids. We see, then, that it is possible to show mathematically that the valency of the diffusible ions will affect the degree of the equation. An unequal distribution of diffusible ions at equilibrium on two sides of a membrane, such as we have above, must result in a difference of electromotive force between the two solutions separated by the membrane. Now this electrical potential difference between the two solutions at equilibrium should be given by the Nernst formula and is equal to $\frac{RT}{F} \log_e \frac{x}{y}$,

where R = the gas constant in electrical units, F = the number of coulombs in a farad of electricity, and T = the absolute temperature. In the case of the system (A) the formula becomes

$$\frac{RT}{F} \log_e \frac{\sqrt{y\,(y+z)}}{y} = \frac{RT}{2F} \log_e \left(1 + \frac{z}{y}\right)$$

and in the case of system (B),

$$\frac{RT}{F} \log_e \frac{\sqrt[3]{y^2\,(y+z)}}{y} = \frac{RT}{3F} \log_e \left(1 + \frac{z}{y}\right).$$

Thus we see that the ratio of the values of the potential difference in system (A) to that in system (B) is 3 : 2.

This potential difference, deducible mathematically as given above, was demonstrated by Loeb in the case of proteins by the use of the method shown in Fig. 6. A

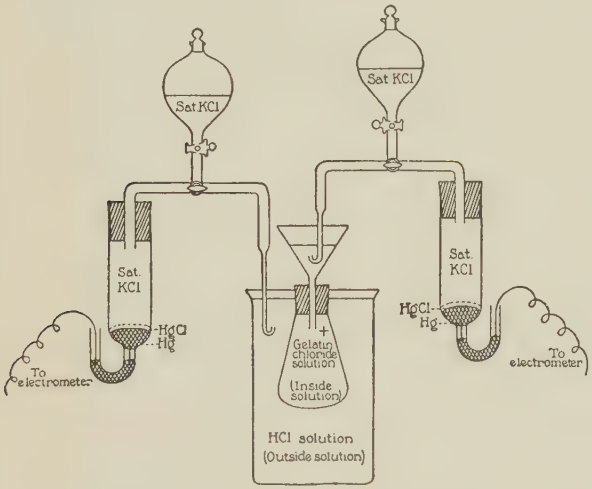

FIG. 6. — Method of measuring the potential difference between gelatin chloride solution in a collodion bag and the outside hydrochloric acid solution in the beaker. (Reproduced from Loeb, "Proteins and the Theory of Colloidal Behaviour," by permission of Messrs. McGraw-Hill.)

collodion bag holding about 50 c.c. of the protein solution is immersed in the dilute acid solution, and after equilibrium has been attained (some eighteen hours at 24°) the membrane potential is obtained by measuring the E.M.F. of the cell :—

Hg	HgCl Sat. KCl	Gelatin Acid Solution	Collodion Membrane	Outside Acid Solution	Sat. KCl HgCl	Hg.

On plotting the observed membrane potentials in millivolts against the p_H of the protein-acid solution it is found that

the values given by acids with monovalent ions, such as hydrochloric, hydrobromic, hydriodic, nitric, acetic, propionic, and lactic acids, all lie on one curve, whilst the potentials obtained with sulphuric and sulphosalicylic acids lie on a common but different curve. Moreover, the curves all show a minimal membrane potential at the isoelectric point of the protein, and all rise rapidly with diminishing p_H until a maximum is reached at about p_H 4·0, and fall again less rapidly with a further diminution in the p_H. The following table illustrates these points :—

TABLE IV

Membrane Potentials for Dibasic and Monobasic Acids

p_H.	Dibasic Acids.	Monobasic Acids.	Ratio $\frac{\text{Dibasic}}{\text{Monobasic}}$.
	Millivolts.	Millivolts.	
2·4	7·6	11·4	0·67
2·6	9·6	14·8	0·65
2·8	11·6	18·0	0·64
3·0	13·6	21·6	0·65
3·2	15·8	24·8	0·64
3·4	18·0	28·0	0·62
3·6	19·8	31·0	0·64
3·8	21·2	34·2	0·62
4·0	21·6	35·5	0·61
4·2	20·8	34·8	0·60
4·4	19·2	31·0	0·62

Here, again, we observe the phenomenon of a minimum point in a physical property of the protein corresponding with the isoelectric point. Further, just as we found the simple valency rule to be valid in regard to the action of ions on the osmotic pressure, viscosity, and other properties of the proteins, so we see that this simple rule applies to the action of anions on the membrane potential. It was shown that on mathematical grounds the ratio of the effect of a

monovalent ion to that of a divalent ion in influencing the membrane potential difference should be 3 : 2, and the above table shows how nearly the experimental results accord with this theoretical requirement, an observation which strikingly confirms the applicability of Donnan's theory of the origin of this potential difference.

Biochemical Application of the Donnan Membrane Equilibrium

Donnan himself was the first to point out that his theory had obvious important applications in biochemistry and physiology, and, following on Loeb's success in utilising the theory to explain the colloidal behaviour of the proteins, it has been applied to such physiological phenomena as the distribution of salt ions between media separated by semi-permeable membranes. Indeed it is obvious that most cell membranes are just such semi-permeable membranes as are required by Donnan's theory. So far most of these applications have been made in studies of the equilibrium existing between red blood cells and plasma, and an investigation made by Barcroft and A. V. Hill and their collaborators may serve as an illustration of the usefulness of these views on membrane equilibria. Here the theory was used to explain why at a given hydrogen ion concentration of the blood plasma, two individuals may show different oxygen-hæmoglobin dissociation curves. The supposition is made that of the ions present in the plasma and corpuscles, Cl, HCO_3, H, OH, phosphates (P), and basic ions (B), all may pass through the membrane surrounding the corpuscle except the last two mentioned. Then from the considerations already advanced (equation A, p. 41),

$$\frac{[H]_1}{[H]_2} = \frac{[Cl]_2}{[Cl]_1} = \frac{[HCO_3]_2}{[HCO_3]_1} = \frac{[OH]_2}{[OH]_1}.$$

or

$$\frac{[H]_1}{[H]_2} = \frac{[Cl]_2 + [HCO_3]_2 + [OH]_2}{[Cl]_1 + [HCO_3]_1 + [OH]_1} \quad . \quad . \quad . \quad . \quad . \quad (B)$$

where the square brackets represent ionic concentrations, and the small numbers $_1$ and $_2$ the two sides of the corpuscular membrane. It is then argued that of the total charge carried by the ions within the corpuscle hæmoglobin can provide but a small part, owing to its great molecular weight, and, therefore, compared with the concentrations of chlorine, phosphate, bicarbonate, and basic ions, the concentrations of the hydrogen and hæmoglobin ions are negligibly small. Hence the negative ions must have their charges balanced almost wholly by positive basic ions, and one may write for the two sides of the membrane

$$(1) \quad [B]_1 = [Cl]_1 + [HCO_3]_1 + [OH]_1 + [P]_1$$

and

$$(2) \quad [B]_2 = [Cl]_2 + [HCO_3]_2 + [OH]_2 + [P]_2$$

and combining these with the equation (B) given above, we have

$$\frac{[H]_1}{[H]_2} = \frac{[B]_2 - [P]_2}{[B]_1 - [P]_1}.$$

It therefore follows that any difference in the concentrations of the basic and phosphate ions on the two sides of the corpuscular membrane must lead to a proportional but inverse inequality in the distribution of the hydrogen ions. Thus we may have an internal corpuscular hydrogen ion concentration different from that of the surrounding plasma, and in two individuals, even if the latter is identical in both, the former need not be so if the basic ion—phosphate ion balance is different. Hence the dissociation curves of the oxyhæmoglobin present in the intact corpuscles of the two individuals may also be different, even if the plasma hydrogen ion concentrations are the same, as Barcroft and Hill found experimentally.

References

1. This chapter is based largely on the work of Loeb and his associates, for a detailed account of which the reader is referred to "Proteins and the Theory of Colloidal Behaviour." J. Loeb. New York and London: McGraw-Hill Book Company, 2nd Edition, 1924.

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CHAPTER III

THE NUCLEO-PROTEINS

In 1868 the substance which we now call nucleo-protein was isolated by Miescher from the nuclei of pus cells. Miescher called his preparation "nuclein," and described it as containing phosphorus and responding to the ordinary colour tests for proteins. It was soon demonstrated by Hoppe-Seyler and by Kossel respectively that a similar substance could be obtained from the nuclei of yeast cells and from avian red blood cells. Later Miescher extended his investigations to the sperm cells of the salmon, material well suited to his object because of the ease with which the nuclei may be separated from the relatively small tail and middle portion of the spermatozöon. From the nuclei thus obtained he isolated a substance which he regarded as a chemical individual, a salt of the basic protein protamine, and an acidic substance which he called nucleic acid. The discovery of these two important constituents of cell nuclei may, therefore, be ascribed to Miescher. Since that time the chemical nature of the nucleo-proteins and their occurrence in such fundamentally important and characteristic structural components of living matter as the cell nuclei has sufficed to keep them in the forefront of the attention of biochemists.

The study of the constitutional aspect of this group of proteins has centred round two main problems—the nature of the association of nucleic acid with protein, and the constitution of nucleic acid itself. With regard to the former problem, it had been supposed that in the cell nuclei the nucleic acid was combined with a basic protein to form a substance called, somewhat vaguely, "nuclein," which latter

had further complex associations with proteins of the histone type, and claims were made from time to time on behalf of the chemical individuality of such nuclein-protein complexes. The position at present is much clearer, and we may now regard the nucleo-proteins of the living cell as consisting of various salts of basic proteins with nucleic acid, in which the former component is in excess. Any method of extraction of the nucleo-proteins, or a process of partial hydrolysis, involves a removal of part of the protein, leaving a mixture of more acidic substances of indefinite composition, and we may regard as such the " nuclein " of the earlier investigators in this field. The present-day position is well expressed by Jones as follows : " The terms nucleo-protein, nuclein, and nucleic acid express a relation which means little more than that conveyed by the terms basic lead acetate, lead acetate, and acetic acid," and " in reality ' nucleo-protein ' means rather a ' method of preparation ' than a chemical substance." Such views are fully borne out by the recent observations of E. Hammarsten, who finds that pure nucleic acid prepared from the thymus gland combines with proteins in varying proportions. The acid has four dissociable hydrogen atoms through which it may effect such combinations, and proteins with an isoelectric point of p_H 4·7 combine with the nucleic acid more readily during the stage of dissociation of the first two of these atoms than during the later stages of the dissociation. The behaviour of nucleic acid in this respect, therefore, conforms to expectations if one regards it as a polybasic acid.

Nucleic Acids

It will be apparent from the foregoing that the chemistry of the nucleo-proteins centres largely round the nature of their specific acidic component, nucleic acid. Modern views tend to the recognition of the existence of but two nucleic acids. One of these is obtainable from the nuclei of plant cells, the other from the nuclei of animal cells. Yeast-

nucleic acid and thymo-nucleic acid (from the thymus gland) will serve as types of these two acids. The terms yeast- and thymo-nucleic acid have been, and are still, widely used as an indication of the source of the nucleic acids, but inasmuch as all plant nucleic acids may be regarded as identical with that of yeast, and all animal acids with that of the thymus cell nuclei, the application of the terms need not be restricted to nucleic acids derived from yeast cells and thymus glands respectively. It is legitimate to refer to the thymo-nucleic acid of the pancreas, but it is perhaps preferable to use the general term "animal nucleic acid." The two nucleic acids closely resemble one another in yielding on ultimate hydrolysis (1) phosphoric acid, (2) a sugar or derivatives of a sugar, (3) bases of the purine and pyrimidine type. They differ, however, in the nature of their sugar residues and in the variety of their bases, as shown in the following table :—

TABLE V

Components of Nucleic Acids

Of Plant Origin.		Of Animal Origin.
Phosphoric acid.		Phosphoric acid.
Pentose sugar (*d*-ribose).		Lævulinic and formic acids derived from a hexose.
Guanine, Adenine	Purine bases	Guanine, Adenine.
Cytosine, Uracil	Pyrimidine bases	Cytosine, Thymine.

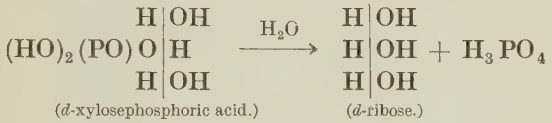
There can be no question concerning the occurrence of one principal nucleic acid in plant and another in animal cell nuclei, and that these two acids have respectively the compositions given above. But the whole question is somewhat more complex than this would indicate. One can isolate from the glandular tissues of animals two substances called inosinic acid and guanylic acid. The latter is obtained by removing the protein from the so-called β-nucleo-protein of Hammarsten. These substances, although simpler than

the nucleic acids proper, are very closely related to them and belong to the class of substances called nucleotides, the nucleic acids being formed, as we shall see, by the association of four nucleotides similar to inosinic and guanylic acids. The study of the simple mono-nucleotides, therefore, serves to elucidate the nature of the more complex nucleic acids. Inosinic acid yields, on hydrolysis, phosphoric acid, the pentose ribose, and the purine base hypoxanthine in equimolar proportions, whilst guanylic acid differs from it only in containing guanine in place of hypoxanthine. The point of interest is that, although these mono-nucleotides are of animal origin and are closely related to the nucleic acids, they contain the pentose which is typical of plant-cell nuclei. There is, however, no reason to associate these two simple nucleotides with the cell nuclei. They appear to be extra-nuclear cell constituents. As a counterpart to this occurrence of pentose nucleotides in animal tissues, an adenine hexoside has been reported from yeast. It cannot be a decomposition product of yeast-nucleic acid, since this contains, as we have seen, a pentose only, and therefore it must have a separate existence in the yeast cell. The view that an absolutely sharp distinction between plant and animal nucleic acids cannot be drawn with regard to their sugar residue is gaining ground amongst biochemists. The presence in animal tissues of the two pentose nucleotides mentioned above has been recognised for some time. More recently the brucine salts of adenine, cytosine (Jones and Perkins), and uracil (Jorpes) pentose nucleotides have been isolated, in addition to guanylic acid, from the mixed nucleotides obtained by hydrolysis of the β-nucleo-protein of pancreas, whilst adenine pentose nucleotide identical with that obtained from yeast-nucleic acid has been separated in a crystalline condition from deproteinised pig blood filtrates (Hoffman). It has also been suggested that the poly-nucleotide of the "coupled" guanyl-nucleic acid, first described by Feulgen and isolated by him from pancreas, is none other than yeast-nucleic acid, which

would account for the isolation of the individual nucleotides mentioned above. It is at least clear from these observations that nucleotides containing pentoses have a considerable distribution in animal tissues. Feulgen and his co-workers have recently described a test, which they call the " nucleal " reaction, and which may be applied to microscopic preparations of tissues for the purpose of detecting the presence of animal-nucleic acid. The tissue is subjected to mild partial hydrolysis with n/10 hydrochloric acid at 60° for four minutes, with the object of liberating the aldehydic groups in the hexose units responsible for the reaction, and the tissue is then treated with fuchsin-sulphurous acid. A positive reaction, which consists in the development of a red-violet colour, is given only by nucleic acids containing hexose units such as we have seen characterise animal nuclei. Nevertheless, tissue preparations from certain of the higher plants, such as wheat embryos and bulbous plants, give a positive reaction, and therefore presumably contain a nucleic acid of the hexose type in addition to the well-recognised pentose acid. Bacteria, like yeast cells, give a negative result, as also do simple protozoa such as trypanosomes, whilst ciliate protozoa (infusoria) give a positive response to the test. From these observations Feulgen concludes that the yeast-nucleic acid is the primitive type, that it is partly replaced in the nuclei of the higher plants by the hexose (animal type) acid, and that it practically disappears in the higher animals, where it survives only as the extra-nuclear pentose mono-nucleotides which we have already mentioned. It is possible, however, that the coupled guanyl-nucleic acid, which contains pentose, and probably consists of yeast-nucleic acid coupled with guanylic acid, is a true nuclear acid. If so, we have a clear case of a plant nucleic acid, and not merely an isolated mono-nucleotide occurring in animal tissues.

The Sugar of Nucleic Acids.—The pentose sugar of plant nucleic acid and of inosinic and guanylic acids has been definitely identified as *d*-ribose.

d-Ribose is found only in plant nucleic acid and its occurence in this source and nowhere else in Nature is unique. Professor R. Robinson, together with Professor Haworth and Dr. E. L. Hirst, suggest that the sugar is in reality the widely distributed *d*-xylose, and that during hydrolytic removal of the phosphoric acid group a Walden inversion takes place thus:—

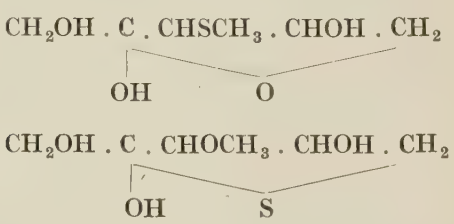


(*d*-xylosephosphoric acid.) (*d*-ribose.)

d-Ribose might similarly arise from *d*-arabinose phosphoric acid and from *l*-lyxose phosphoric acid, but inasmuch as neither *d*-arabinose nor *l*-lyxose are to be found in Nature, the *d*-xylose theory is to be preferred. Great importance attaches to these views regarding optical inversion during hydrolytic removal of phosphoric and similar groups from sugar residues and the subject is dealt with further in Chapter V.

On ultimate hydrolysis of animal nucleic acid, lævulinic and formic acids are obtained in place of a free hexose. On oxidation with nitric acid, Steudel has claimed that the sugar residue is obtained as a dicarboxylic acid of the type of saccharic acid. Feulgen has suggested that no hexose as such is present, but an unsaturated sugar of the nature of glucal. Steudel and Peiser ascribe the reactions on which Feulgen based this view, including the fuchsin-sulphurous acid test, to furfural. But Feulgen has pointed out that the fuchsin test is not given by furfural and is absolutely specific for the partly hydrolysed animal nucleic acid. Indeed, if the fuchsin test was due to furfural, it is difficult to see why it should not be given by plant nucleic acid, bearing in mind the readiness with which the aldose pentoses yield furfural on treatment with hydrochloric acid. Feulgen's most recent conclusion is that the carbohydrate residue is not a hexose,

but a related substance of unknown constitution. Meanwhile a possible new line of investigation seems to be opening out. Mention has been made of an adenine hexoside which was isolated from yeast (p. 51). This substance on hydrolysis yields lævulinic acid. Suzuki recently made the interesting discovery that the sugar residue contained sulphur, and more recently still Levene and Sobotka have identified the sugar as a methyl ketopentose containing sulphur. The sulphur is not present as a sulphydryl group, but the presence of either an OCH_3 or a SCH_3 group has been demonstrated. Levene and Sobotka suggest one of the following structural formulæ for this interesting substance, the position assigned to the methoxyl or thiomethoxyl group being arbitrary :—



The possibility that some such similar substituted ketopentose sugar, not necessarily containing sulphur, may constitute the sugar of the animal nucleic acid seems worthy of consideration, especially in view of the repeated failures to characterise this definitely as a hexose. But it is obvious that much further investigation is required to clear up this difficult question.

The Structure of Inosinic and Guanylic Acids and their Relation to Plant Nucleic Acid

On hydrolysis with acids inosinic acid decomposes according to the equation :—

$$\underset{\text{(Inosinic acid.)}}{C_{10}H_{13}O_8N_4P} + 2\,H_2O \longrightarrow \underset{\text{(Ribose.)}}{C_5H_{10}O_5} + H_3PO_4 + \underset{\text{(Hypoxanthine.}}{C_5H_4ON}$$

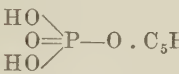
It is, however, possible to adjust the conditions of hydrolysis so that intermediate products may be isolated. Thus mild acid hydrolysis yields a pentose phosphate and hypoxanthine, whilst neutral hydrolysis under pressure yields phosphoric acid and the nucleoside inosine, a compound of ribose and hypoxanthine of the formula $C_{10}H_{12}O_5N_4$. These results can only be accommodated in a structural formula of inosinic acid which places the ribose unit in the central position between the phosphoric acid and the hypoxanthine, thus :—

INOSINE
neutral hydrolysis

PHOSPHORIC ACID———RIBOSE———HYPOXANTHINE

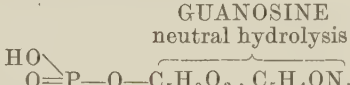
acid hydrolysis
PENTOSE PHOSPHATE

The constitution of inosinic acid is therefore



Similar observations serve to establish the constitution of guanylic acid as a mono-nucleotide in which phosphoric acid and the purine base guanine are linked through ribose, thus :—

GUANOSINE
neutral hydrolysis



(Guanylic acid.)

On applying to yeast-nucleic acid the method of neutral hydrolysis under pressure the four nucleosides given below are obtained together with four molecular proportions of phosphoric acid. The course of further hydrolysis of these nucleosides is also included in the scheme.

(1) Guanosine	——→	*d*-ribose + guanine	Purine
(2) Adenosine	——→	*d*-ribose + adenine	bases.
(3) Cytidine	——→	*d*-ribose + cytosine	Pyrimidine
(4) Uridine	——→	*d*-ribose + uracil	bases.

On the basis of these results Levene and Jacobs suggested that yeast-nucleic acid is composed of four mono-nucleotides, each formed by the addition of phosphoric acid to the nucleosides obtained as above. In confirmation of this hypothesis they prepared from yeast-nucleic acid the four expected nucleotides of the constitution :—

$$\begin{matrix} HO \\ O \\ HO \end{matrix} \!\!>\!\!= P — O \,.\, C_5H_8O_3 \,.\, B,$$

B representing the bases guanine, adenine, cytosine, and uracil. This hydrolysis of yeast-nucleic acid into its component nucleotides is effected by the action of a thermostable agent present in a boiled aqueous extract of the pancreas of the pig,[1] or by the action of ammonia at 105° to 115°. The relative ease with which the further hydrolysis of these individual nucleotides is effected is different in the two types of nucleotide. Thus the phosphoric acid is liberated from the pyrimidine nucleotides at a much slower rate than is that of the purine nucleotides. This difference in the behaviour of the nucleotides is illustrated by Jones as follows :—

Ten portions of commercial yeast-nucleic acid from the same specimen are weighed into appropriate vessels, and in one of these the total phosphoric acid is determined. The other nine portions are submitted to hydrolysis with 5 per cent. sulphuric acid at 100° for various periods from one-quarter to seven and a half hours, and after removal of the guanine with ammonia, the phosphoric acid is precipitated with magnesia mixture and weighed as magnesium ammonium phosphate. The weight of magnesium ammonium phosphate obtained in each experiment is divided by the weight of nucleic acid from which it was produced, and from the comparable results so obtained the upper curve (A) in Fig. 7 is constructed. The rapid initial rise in the curve, its

[1] There now appears to be some doubt as to the power of the thermostable agent to liberate all the component nucleotides of yeast-nucleic acid.

sharp turn, and termination in a straight line slightly inclined to the abscissa show clearly that in the acid hydrolysis of yeast-nucleic acid phosphoric acid is progressively liberated from two sources. From the one the production is rapid and complete in less than two hours ; from the other it is much slower, continues beyond the time consumed in these experiments, and is so regular that its amount is definitely seen to be equivalent to 10 mg. of magnesium ammonium phosphate per hour. If we make a deduction for this amount from the values recorded in the first curve, we obtain the lower

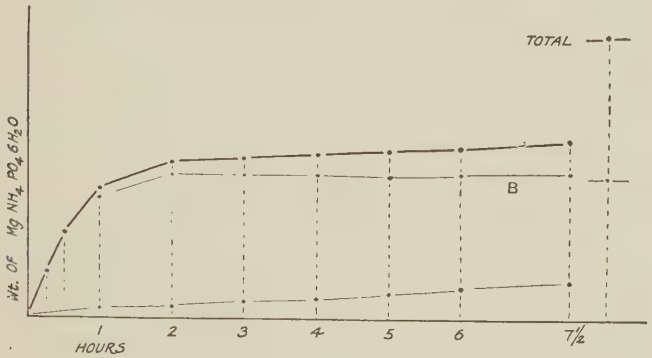
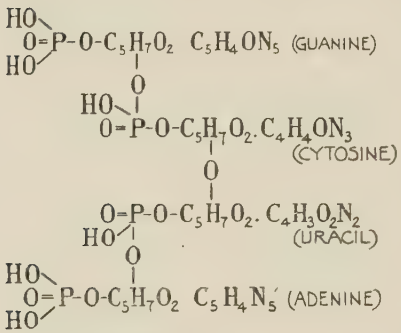


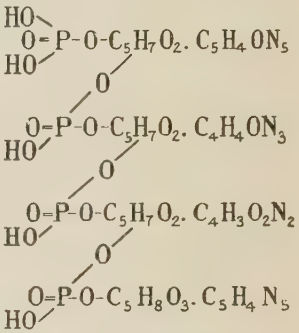
FIG. 7.—Rate of liberation of phosphoric acid from yeast-nucleic acid. (After Jones.)

curve (B) in Fig. 7. This curve ends in a straight line parallel to the abscissa whose ordinate corresponds to half the total phosphorus obtainable from the nucleic acid ; or, in other words, the phosphoric acid which is rapidly and completely liberated by acid hydrolysis of yeast-nucleic acid is half the total. It is possible to show that at the point where the curve A makes its sharp turn the purines have been completely set free whilst the pyrimidines are still combined. It is obvious that the two sources of phosphorus are the purine nucleotides and the pyrimidine nucleotides, and as these sources are equal in amount the purine groups of yeast-nucleic acid must be equal in number to the pyrimidine

groups. In confirmation of these views it is found that, in the cases of the free nucleotides of guanine and adenine, the "easily split" phosphorus is equal to the total, and both give a hydrolysis curve practically coincident with curve B of Fig. 7. Further, a mixture of equivalent quantities of adenine nucleotide and uracil nucleotide gives off half of its phosphoric acid easily and holds the other half much more firmly, whilst a mixture of cytosine and uracil nucleotides under similar conditions gives off an amount of phosphoric acid which corresponds to only 10 mg. of magnesium



JONES' FORMULA



LEVENE'S FORMULA

ammonium phosphate per hour, as shown in the inclined straight line in Fig. 7. The most obvious explanation of the foregoing observations is that yeast-nucleic acid is composed of two purine nucleotides and two pyrimidine nucleotides, all of which contain phosphoric acid and ribose, whilst each contains a different base. The method by which these four nucleotides are linked together still remains to be discussed. In their earlier observations Jones and his associates failed to demonstrate any increase in the acidity of a digest of yeast-nucleic acid with the thermostable agent in a boiled extract of pig's pancreas, which, as we have seen, splits the nucleic acid into its component nucleotides. If the

hydroxyl groups of the phosphoric acid residues were involved in the inter-nucleotide linkage, one might expect a marked increase in the acidity of the digest during the progress of the hydrolysis. But more recent observations by Jones and Perkins, in which a quantitative conversion of yeast-nucleic acid into its mono-nucleotides was achieved by the action of 1 per cent. sodium hydroxide at room temperature, show a progressive diminution in the alkalinity as decomposition proceeds, indicating the liberation of weak acids. The earlier view that the phosphoric acid groups played no part in the linking has, therefore, been abandoned, and it is now supposed that there are present two linkings between phosphoric acid and ribose of an ester type, and another linking of an ether type between the other two central ribose molecules. Jones and other workers exclude the possibility of the bases participating in the linking, largely because the rapid rate of liberation of the purines and the slow rate of liberation of the pyrimidines from the parent acid are the same as those observed in the cases of the free nucleotides. Levene, on the other hand, supposes that all three linkings are effected through a pentose and a phosphoric acid group. The whole question of the internal linkings of yeast-nucleic acid must still be regarded as open, but the two possibilities mentioned are given on p. 58. An interesting consequence of acceptance of the theory already discussed (p. 53), regarding the nature of the sugar residue of yeast-nucleic acid, is that the phosphoric acid groups should be attached to the pentose residues at position 3.

In a recent study of the dissociation constants of the individual nucleotides and nucleosides of plant nucleic acid, Levene and Simms adduce important evidence in favour of the three ester linkings as opposed to the suggestions of Jones.

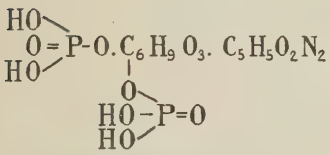
None of the formulæ yet proposed gives a really satisfactory explanation of the marked difference in the rate of further decomposition of the pyrimidine as contrasted with the purine nucleotides.

The formulæ given above as representing plant nucleic acid may stand in need of further correction. Using the method of cold hydrolysis with dilute sodium hydroxide recently devised by them, Jones and Perkins have obtained from this acid guanine nucleotide, adenine nucleotide, large quantities of cytosine nucleotide, but no uracil nucleotide. They likewise obtain very small yields of the latter on hydrolysing the nucleic acid with the thermostable component in an extract of pig's pancreas. They therefore conclude that uracil does not exist preformed in the nucleic acid molecule, but arises during hydrolytic cleavage by secondary decomposition of cytosine. This explanation of the occasional presence of uracil in the hydrolysis products of animal nucleic acid has now been held for some time. Thus it would seem that uracil is not present as such in either of the two nucleic acids.

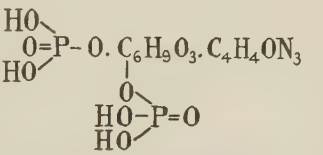
Animal Nucleic Acid

The ease with which plant nucleic acid is decomposed into its four component nucleotides by the action of ammonia at 105° to 115° is not paralleled by the behaviour of the animal acid, nor does the latter yield its purines in an identifiable form as readily as does the plant acid on hydrolysis with dilute mineral acid. There is, however, no doubt regarding the close constitutional similarity of the two nucleic acids apart from the differences in the nature of their ultimate hydrolytic products as given on p. 50. Four of these products are identical (phosphoric acid, guanine, adenine, and cytosine), whilst the other two are closely related. These observations and the facts that a thymine nucleotide, a guanine nucleoside, and various other intermediate decomposition products similar to those obtained from the plant acid have been prepared from the animal nucleic acid, support the view that the latter is constituted on lines closely similar to those of the plant acid, *i.e.*, from four nucleotides. This is borne out by the recent analyses of pure preparations of

nucleic acid obtained by Levene from the thymus gland, spleen, kidney, pancreas, and liver. All of these preparations have the elementary composition of a tetra-(hexose)-nucleotide. With regard to the mode of internal linking of the component nucleotides, discussion has centred round the question of the presence of an ether or of an ester type of oxygen linking. Jones and Tannhauser have favoured the former view, whilst Levene supports the latter. On hydrolysing animal nucleic acid with 2 per cent. sulphuric acid Levene and Jacobs obtained two compounds, to which they ascribed the constitutions of cytosine and thymine nucleotides, each carrying one additional phosphoric acid group as shown in the accompanying formulæ :—



HEXO-THYMIDINE-DIPHOSPHORIC ACID



HEXO-CYTIDINE-DIPHOSPHORIC ACID

They also claimed to have isolated a dinucleotide containing the two pyrimidine residues. In later work Levene could obtain only the two former compounds, the barium salt of thymidine-diphosphoric acid being prepared in a crystalline form. Leaving out of consideration the undecided question of the possible existence of a dinucleotide in the hydrolysis products of the animal acid, Levene explains his results on the basis of three ester linkings each between a sugar unit and a phosphoric acid residue. The structure proposed is, therefore, similar to that suggested for the plant acid, but, as in the instance of the latter acid, it cannot be said that the question is definitely settled. Such differences of behaviour as do exist between the two acids must on these views be ascribed to the sugar components.

To summarise, we may classify the nucleic acids and related compounds as follows :—

I. *Nucleic Acids containing Pentoses.*

(1) Mono-nucleotides. (*a*) Inosinic acid.
(*b*) Guanylic acid.

(2) Poly-nucleotides. Plant nucleic acid.

II. *Nucleic Acids* which, after splitting off purines, give a violet reaction with fuchsin-sulphurous acid and a green pine shaving reaction, and which probably contain a readily decomposable hexose or other unidentified sugar residue.

Animal nucleic acid.

III. A class of *Mixed Nucleic Acid* showing the reactions of both groups I. and II., and possibly consisting of a mechanical mixture or loose combination of the members of these groups.

Nucleic Acid Metabolism

The metabolic transformations of the nucleoproteins, as revealed both by those ingested in the foodstuffs and by those arising from the endogenous processes of tissue breakdown, provide an exquisite example of a co-ordinated sequence of specific enzymic activities. It is perhaps regrettable that the biochemist must give expression to these phenomena by postulating the existence of an elaborate profusion of distinct, but as yet unisolated, enzymes. At the present day our knowledge of biochemical processes appears to offer no other alternative. In the meanwhile it may perhaps help towards an understanding of the realities behind the names given to these hypothetical enzymes if we regard the names themselves as summarising in a definite expression the capacity of the tissues concerned to bring about the given chemical transformations, without necessarily adopting the view that a distinct enzyme is essential for each different chemical reaction or committing ourselves

to definite views concerning the mechanisms of the transformations.

Dealing first with the exogenous aspect of the metabolism of these substances, one finds that the protein portion of the nucleoproteins ingested in the alimentary tract is removed in the course of gastric digestion, and by the action of the pancreatic juice, trypsin being much more effective than pepsin in this respect. There seems no reason to doubt that

TABLE VI

The Fate of Purines injected Subcutaneously in the Monkey

Substance.	Purine N. Administered.	Recovered Nitrogen.		Bases.	Total.	Per Cent. Recov.
		Allantoin.	Uric Acid.			
	mg.	mg.	mg.		mg.	
Uric acid . .	36	8	14	—	22	61
Uric acid . .	49	12	23	—	35	71
Guanine * . .	71	38	2	20	60	99–105
Xanthine . .	56	35	2	17	54	97
Adenine * . .	64	15	3	21	39	69–76
Hypoxanthine .	50	28	3	9	40	80
Sodium nucleate * .	90	38	3	26	69	88–96

* In these cases the uric acid and allantoin produced contain but four-fifths of the original amino-purine nitrogen, and the lower figure for percentage recovery is based on the assumption that the extra bases are amino-purines, whilst the higher takes them as oxy-purines.

the alimentary enzymes are capable of completely digesting the protein moiety. There is, however, no convincing evidence to show that the nucleic acid residue is attacked by either the gastric or pancreatic enzymes. The exclusion of pancreatic juice from the intestine does not affect the course of nucleic acid digestion. On the other hand, the *succus entericus* and the intestinal mucosa do contain enzymes which effect a considerable disintegration of the nucleic acid molecule. The results of Levene, of London, and of Jones and

their co-workers, concerning this question may be summarised as follows : (*a*) No digestion or absorption of nucleic acid occurs in the stomach ; (*b*) Extirpation of the stomach or the pancreas does not influence nucleic acid digestion ; (*c*) Intestinal juice (*succus entericus*) contains a *nucleinase* which hydrolyses tetra-nucleotides into mono-nucleotides, and *nucleotidases* specific for the purine nucleotides, which latter may be hydrolysed into phosphoric acid and nucleosides, or into purine base and sugar phosphate. The pyrimidine nucleotides do not appear to be affected by the intestinal juice ; (*d*) Extracts of the intestinal mucosa contain a still wider range of enzymes. There is present in these a nucleinase, nucleotidases for both purine and pyrimidine nucleotides, and *nucleosides* specific for the purine nucleosides which are hydrolysed into sugar and base. The pyrimidine nucleosides appear to resist hydrolysis both in the intestine and in the intestinal wall, behaviour which is parallel to their resistance to hydrolysis *in vitro*. It is, therefore, probable that, as a result of the progressive removal of the protein from the nucleoprotein, and the subsequent action of the extra- and intra-cellular enzymes of the intestinal mucosa, the products arising from the disintegration of the nucleic acid enter the portal circulation in the form of phosphoric acid, nucleosides (principally pyrimidine), some sugar, and guanine and adenine.

The subsequent fate of these fractions—and investigations have been largely confined to the purine bases—is well shown in a series of experiments by Hunter and Givens, in which various purines were administered subcutaneously to the monkey. Some of their results are given in Table VI. on p. 63.

From these results we see that the free purines are catabolised, in general, in the same way as the combined purines of nucleic acid, giving rise to uric acid and allantoin as final products. The relatively smaller amount of nitrogen recovered after uric acid administration is interesting, and

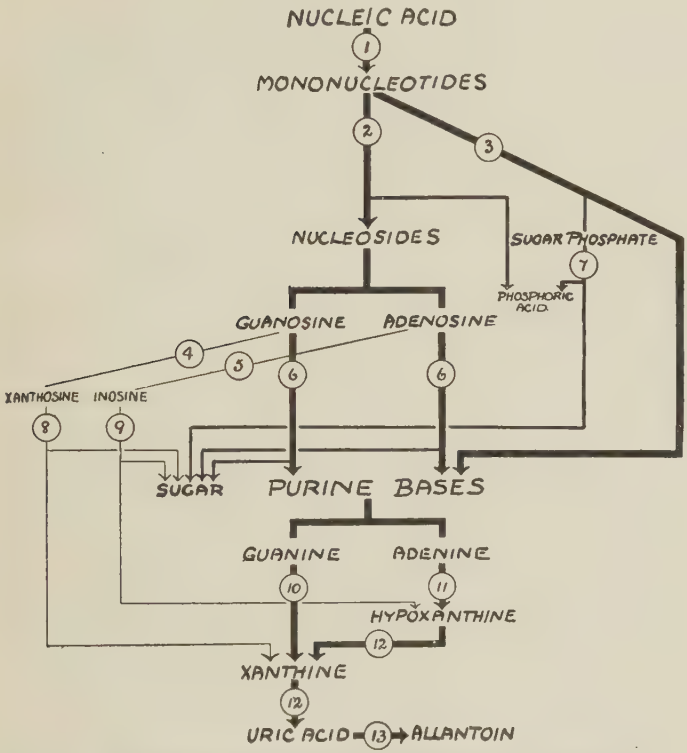


FIG. 8.—Schematic representation of the catabolism of nucleic acid. The pyrimidine bases are not included. The enzymes which are responsible for the various changes and to which the numbers refer are given in the following list:—

(1) Nucleinase.
(2) Nucleotidase or phospho-nuclease, active in acid solutions.
(3) Nucleotidase or purine-nuclease, active in alkaline solutions.
(4) Guanosine-deaminase } These enzymes permit of a possible deaminisation of the nucleosides before they are completely hydrolysed.
(5) Adenosine-deaminase }
(6) Nucleosidases (Guanosine- and Adenosine-hydrolase).
(7) Sugar-phosphatase.
(8) Xanthosine-hydrolase. } Types of nucleosidases.
(9) Inosine-hydrolase. }
(10) Guanase } Deaminising enzymes.
(11) Adenase }
(12) Xanthine-oxidase.
(13) Uricase, not found in man and the higher apes.

will be referred to later. The general view adopted at the present time is that the catabolism of both exogenous and endogenous nucleic acid products follows identical courses. The succession of enzymes responsible for these metabolic transformations and the changes which each one brings about have been summarised in the scheme given (Fig. 8) on p. 65, to which the reader is referred. Most of the enzymes appear to have wide distributions throughout animal tissues, but species are known in which one or other of the enzymes mentioned are lacking in an organ in which it is known to occur in another species. Thus xanthine oxidase appears to be entirely absent from the tissues of the rat, and whilst ox liver will form uric acid from both adenine and guanine, pig's liver will do so only from adenine, rabbit's liver only from guanine, and dog's liver from neither. One of the most interesting cases of differential distribution of the purine enzymes is provided by the last stage of the oxidations shown on p. 65, namely, the conversion of uric acid to allantoin which occurs in most animals prior to excretion. This further breakdown of uric acid occurs in all mammals with the striking exceptions of man and the higher apes. The oxidation is brought about by the presence of the enzyme uricase, found predominantly in the liver, but occasionally in the spleen and kidney. The extent to which uric acid undergoes this final oxidation in various animals is indicated by the " uricolytic index," which represents the percentage ratio of allantoin nitrogen to the sum of allantoin and uric acid nitrogen excreted. Hunter and his co-workers have determined this index for many animals, and some of their results (quoted by Rose) are embodied in Table VII. on p. 67.

Apart from the unique position of man and the chimpanzee in the above list, the very low index of the Dalmatian coach dog is noteworthy. This curious metabolic discovery was first made by Benedict, who showed that on a purine-free diet this breed of dog excretes about as much uric acid as

man. Uricase is present in the animal's liver. Onslow, in an interesting series of breeding experiments in which Dalmatians were crossed with other dogs, found that the crosses showed the same high uricolytic index as the other dogs. The peculiar Dalmatian characteristic was lost, and there-

TABLE VII

Uricolytic Indices of Various Animals.

Animal Species.	Order.	Uricolytic Index.
Opossum	Marsupialia	79
Rat	Rodentia	96
Mouse	,,	98
Guinea-pig	,,	94
Rabbit	,,	95
Cat	Carnivora	97
Dog	,,	98
Dalmatian coach dog	,,	32
Cow	Ungulata	93
Horse	,,	88
Sheep	,,	80
Goat	,,	92
Pig	,,	98
Elephant	Proboscidea	72
Monkey	Primates	89
Chimpanzee	,,	0
Men	,,	0

fore the presence of uricase in such amounts and activity as to convert most of the uric acid to allantoin is a Mendelian dominant character. In a series of investigations of the purine metabolism of the Dalmatian dog, Benedict found that thymus feeding produced a much greater increase in the amount of allantoin excreted than of uric acid, *i.e.*, the value of the uricolytic index was considerably increased above its

normal value. On administering a subcutaneous injection of uric acid again, a marked increase in the excretion of allantoin was noted despite the fact that the uric acid injected was recovered unchanged in practically quantitative amount. Reference to the results of Hunter and Givens on p. 63 will show that the injection of uric acid produced in the monkey but a slight increase in allantoin and a marked increase in uric acid excretion, although the monkey has a high uricolytic index. The expectation would naturally be that most of the uric acid should be oxidised to allantoin. These anomalous results may be interpreted either in the sense that different metabolic paths exist for endogenous and exogenous purines, or that the production of uric acid or allantoin are inter-related in unknown ways. The balance of evidence is against the former alternative, as we have seen, and at present one can but record these observations as indicating fields in which further investigations are required.

The Synthesis of Purines in Animal Organisms

Abundant evidence exists to show that the animal organism is capable of synthesising purines, and ultimately nucleic acid and nucleo-proteins. We shall see in a later chapter that the disappearance of part of the phospho-protein phosphorus in the incubating hen's egg is associated with a formation of nucleo-protein. The necessary purines do not exist preformed in the unincubated egg, but they appear soon after incubation has commenced and progressively increase during the period of incubation. During the migration of the salmon to its spawning grounds, a period in its life cycle when it abstains from food, relatively enormous amounts of nucleic acid are formed in the spermatozoa, the material for this synthesis arising from the disintegration of other tissues, notably the muscle tissue. That this does involve a synthesis of purines is proved by the relatively small amounts of preformed purines present in the muscle.

Carefully controlled experiments on rats, rabbits, pups, dogs, and on adult man conclusively prove that these animals are independent of a source of preformed purines. In one experiment Benedict kept a Dalmatian dog on a purine-free diet for a year, and during this period it excreted more than 100 grams of uric acid, of which it was estimated that not more than 10 per cent. could arise from the preformed purines of the animal's tissues. An interesting illustration of the ability of the animal to synthesise purines is provided by the birds and reptiles. In these animals the larger part of the uric acid excreted represents not purine metabolism, but the normal exogenous protein metabolism. Uric acid takes the place of urea as the end-product of exogenous nitrogen metabolism. The fact that the uric acid molecule may be regarded as being composed of two urea molecules linked together by a chain of three carbon atoms lends support to the view that in the bird and reptile uric acid may be synthesised from urea. In any case the ability of these animals to achieve the synthesis from non-purine nitrogen compounds is undoubted. Suggestive experiments by Ackroyd and Hopkins point to both histidine and arginine being possible and independent sources of purines in the mammal. A recent reinvestigation of this question by Rose and Cox gave results in support of this view so far as histidine was concerned, but they were unable to find that normal growth in rats followed the administration of arginine alone to a diet otherwise sufficient, but devoid of histidine. The failure of growth on a diet lacking histidine might not have been due to the absence of a suitable source of purines for nucleic acid synthesis. Indeed, the question of the formation of purines from either of these two amino acids must still be regarded as open. Recent experiments by Stewart, in which surviving excised cats' livers were perfused with aerated Ringer's solution containing histidine or arginine, failed to demonstrate any formation of allantoin, the normal end-product of purine oxidation in the cat.

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CHAPTER IV

THE RÔLE OF TYROSINE

In animal biochemistry an " essential " amino acid is one which is necessary for the normal development, maintenance, or functioning of the organism, and which the latter cannot synthesise for itself from simpler components. It is a reasonable assumption that such an essential amino acid, which therefore must be supplied to the animal as a component of the dietary proteins, is utilised for the formation of some particular tissue or tissue product which in turn exercises a necessary and indispensable control over the animal mechanism. The essential amino acid is, as it were, a " key " substance, and just as " key " industries are regarded as essential to the economy of the body politic so is the " key " substance essential to the animal economy.

One of the essential amino acids is tyrosine, *p*-hydroxyphenylalanine. It has been obvious to biochemists for many years that the hormone adrenaline is derived from tyrosine although the actual steps of the conversion process *in vivo* have not yet been elucidated. It may therefore be argued that one possible explanation of the essential nature of tyrosine is its necessity for the synthesis of adrenaline, but until very recently this has been the sole instance which it has been possible to cite in explanation of the indispensable nature of tyrosine. Recent work now makes it seem probable that this particular amino acid is destined to play an increasingly important *rôle* as a mother substance from which essential tissue products are elaborated. The brilliant researches of Harington on the constitution and synthesis of thyroxine, the principle of the thyroid gland, leave

little doubt that this potent internal secretion is also to be regarded as a derivative of tyrosine, although here again we are still in ignorance of the biochemical process of conversion of tyrosine into thyroxine. On the other hand the work of Raper and his school on the action of the enzyme tyrosinase, one of the oxidases, has revealed step by step much of the mechanism underlying the conversion of tyrosine into the important and widespread animal pigment melanin. Raper's work constitutes in many respects one of the best documented studies so far published of the intermediate action of an enzyme. It is proposed in this chapter to deal with these two fields—the chemistry of thyroxine and the tyrosinase-tyrosine reaction.

The Chemistry of Thyroxine

The announcement of the isolation of crystalline thyroxine was first made by Kendall in 1914. This substance contains some 65 per cent. of iodine, and is unquestionably the compound, or one of the compounds, by means of which the thyroid gland exercises its potent effect on the metabolic rate. In 1919 Kendall and Osterberg assigned the formula $C_{11}H_{10}O_3NI_3$ to thyroxine and ascribed to it the constitution of a tri-iodoindolepropionic acid. On the basis of this constitution thyroxine was regarded as a derivative of the amino acid tryptophane, itself one of the essential amino acids. In 1925 Harington described the synthesis of 3 : 4 : 5-tri-iodophenylpyrrolidone carboxylic acid, a substance which ought closely to have resembled thyroxine according to Kendall's suggestions, but the synthetic product proved to be quite devoid of any physiological action resembling that of thyroxine, and Harington was led to regard the suggested indolepropionic acid formula for thyroxine as improbable. He resolved therefore to isolate the substance himself from fresh thyroid glands in order to obtain material sufficient for a detailed investigation. When Kendall originally isolated thyroxine in a crystalline state

he obtained some 33 grams of the substance from three tons of the fresh glands. This statement alone shows how difficult and tedious the whole operation must have been, and great credit is due to Kendall for carrying it to a successful issue, and for demonstrating that the essential principle of the thyroid gland is a crystalline substance capable of isolation by ordinary chemical means. When Harington repeated Kendall's method of separation of thyroxine he found the process uneconomical and wasteful, and therefore devised a new means of isolating the substance. Harington's process proved to be a considerable improvement on that of Kendall, since he obtained some 0·027 per cent. of the weight of the fresh gland (or 0·12 per cent. of the dried gland) in the form of thyroxine, whilst Kendall's yield was of the order 0·001 per cent. of the fresh gland. In Harington's process some 14 per cent. of the total iodine content of the fresh gland was isolated in the form of crystalline thyroxine. Briefly the process employed by Harington for isolating thyroxine is as follows :—

500 gms. of dried thyroid gland are boiled for five to six hours under reflux with 5,000 c.c. of 10 per cent. barium hydroxide. After standing overnight the heavy, dark precipitate which forms is filtered, and the clear yellow filtrate is acidified with hydrochloric acid. A light-coloured, flocculent precipitate forms and after some hours this is separated, washed with water, dissolved in 250 c.c. of ammoniacal water, treated with sufficient barium hydroxide to make a 40 per cent. solution, boiled, and heated for eighteen hours on a steam bath. The precipitate is separated and washed, ground, and suspended in 250 c.c. of 1 per cent. sodium hydroxide. The solution is boiled and treated with a strong solution of sodium sulphate in slight excess, and after a further few minutes boiling the barium sulphate is separated by filtration. The filtrate is boiled once more and acidified while boiling by an appropriate addition of 50 per cent. sulphuric acid. The precipitate which forms is at first flocculent but becomes heavy and granular, and at this stage boiling is discontinued. After cooling the precipitate is separated and dissolved in 20 c.c. of *N* sodium

hydroxide, alcohol is added to 80 per cent. concentration, and after again filtering to remove a small amount of tarry matter, the solution is boiled and 10 c.c. of 35 per cent. acetic acid are added. Crude thyroxine—about 0·4 gm.—separates in a partially crystalline condition, representing a yield of approximately 0·08 per cent. By working up the heavy dark precipitate first separated a further quantity of crude thyroxine is obtained raising the yield to 0·125 per cent. Crude thyroxine is purified by solution in alkaline alcohol and reprecipitation with acetic acid. The precipitate is then dissolved in boiling 0·5 per cent. sodium carbonate, and on cooling a heavy, white, microcrystalline precipitate of the sodium salt of thyroxine separates. This is centrifuged from the mother liquor, dissolved in 80 to 90 per cent. alcohol containing a little sodium hydroxide, and the boiling solution is treated with 35 per cent. acetic acid in excess. Pure thyroxine is obtained as a perfectly white substance crystallising in rosettes and sheaves of fine needles. It darkens at 220° and melts with decomposition and evolution of iodine at 231° to 233°.

With this carefully purified material at his disposal Harington established the correct empirical formula of thyroxine as $C_{15}H_{11}O_4NI_4$, and that it was indeed the active principle of the thyroid gland that had been isolated was demonstrated in a physiological test in which 10 mg. of the crystalline substance were administered intravenously to a normal individual with a basal metabolic rate of −5 per cent. On the fifth day this had risen to + 40 per cent., whilst the pulse rate had risen from 72 to 120. The effects continued to be evident for a fortnight after the injection.

The next stage in Harington's investigations was directed towards the elucidation of the structure of thyroxine, and his first step was the removal of the iodine atoms leaving the rest of the molecule, if possible, intact. This was accomplished by using a catalytic reducing agent—palladium hydroxide-calcium carbonate. A 1 per cent. solution of thyroxine in normal potassium hydroxide was shaken in an atmosphere of hydrogen in presence of this catalyst. This treatment yielded the iodine free substance—desiodo-

thyroxine—and at the same time it was ascertained from a measurement of the hydrogen uptake that each atom of iodine was replaced by one of hydrogen and that secondary reductive changes were excluded. Desiodothyroxine has the composition $C_{15}H_{15}O_4N$, it gives Millon's and the ninhydrin reactions, and loses all its nitrogen in contact with nitrous acid in the van Slyke apparatus. It is amphoteric in its behaviour to acid and alkali. These observations led to the conclusion that desiodothyroxine is an α-amino acid with at least one phenolic group. The proportions of C and H suggested to Harington the presence of two benzene rings. This was confirmed by the isolation of *p*-hydroxybenzoic acid and quinol, together with ammonia and oxalic acid on subjecting desiodothyroxine to various conditions of alkaline fusion. In addition a substance $C_{13}H_{12}O_2$ possessing one phenolic group and an inert oxygen atom was isolated. Exhaustive methylation and subsequent degradation of desiodothyroxine confirmed its identity as an amino acid, whilst the presence of a three carbon side chain, suggested by the isolation of oxalic acid described above, was proved. The presence of only one phenolic group was likewise confirmed. Desiodothyroxine therefore, may be formulated as $HO.C_{12}H_8O.CH_2.CHNH_2.COOH$, and the remaining question is the nature of the two benzene ring residue represented as $C_{12}H_8O$ in this formula. At this stage Harington formed the supposition that the two benzene rings are linked together through the oxygen atom, and that the grouping $C_{12}H_8O$ represents diphenyl ether minus two hydrogen atoms. The properties of the compound $C_{13}H_{12}O_2$ isolated from the products of alkaline fusion of desiodothyroxine lent support to this view. Harington therefore decided to attempt the synthesis of a compound of the constitution which he supposed desiodothyroxine to possess according to the views set forth in the foregoing, and thus to endeavour to meet the results of his degradation by synthetical experiments. There is no need to give in detail

here the steps employed in the synthesis of desiodothyroxine. It is sufficient to state that it was successfully accomplished and the product obtained, having the constitution

was in every respect identical with that obtained from natural thyroxine by catalytic hydrogenation.

For those readers desirous of obtaining further information regarding this highly interesting synthesis it may be added that the successive steps were : *p*-bromoanisol + potassium phenate ——→ (4′-methoxyphenoxy)benzene ——→ 4-(4′-methoxyphenoxy)benzaldehyde + glycine anhydride ——→ condensation product ——→ (using red phosphorous and hydriodic acid as hydrolytic, demethylating, and reducing agent) ——→ 4-(4′-hydroxyphenoxy)phenyl α-aminopropionic acid, *i.e.*, desiodothyroxine. Alternatively 4-(4′-methoxyphenoxy)benzaldehyde + hydantoin ——→ (using red phosphorous and hydriodic acid) ——→ desiodothyroxine.

These experiments in the degradation of thyroxine, and in the synthesis of desiodothyroxine therefore establish the identity of the former as the tetraiodo derivative of β-[4–(4′–hydroxyphenoxy)phenyl]-α-aminopropionic acid. While the experiments described in the foregoing were in progress, Dakin in America, unknown to Harington, had come to similar conclusions regarding the constitution of thyroxine, and had further observed that on treatment with fuming hydriodic acid at 140° tyrosine was obtained. These important experiments have not been published, but they lend strong and independent support to Harington's views.

There remained to be determined the orientation of the four iodine atoms, and this was finally achieved in the following series of observations. Methylation and reduction of thyroxine yields a tetraiodo derivative of the acid $CH_3O.C_6H_4.O.C_6H_4.COOH$ in which the original iodine atoms remain *in situ*. On condensing quinol monomethyl

ether with 3 : 4 : 5 tri-iodonitrobenzene there is obtained 3 : 5-di-iodo-4-(4′-methoxyphenoxy)nitrobenzene (Formula I). By reduction the corresponding aniline is obtained and subsequently by Sandmeyer's reaction the nitrile (II.). This nitrile on being boiled with a mixture of hydriodic and acetic acids undergoes simultaneous hydrolysis and demethylation to yield 3 : 5-di-iodo-4-(4′-hydroxyphenoxy)benzoic acid (III.). On adding iodine in potassium iodide to a solution of this acid in ammonia, iodine is rapidly taken up to the extent of two atoms, and there is obtained the new compound 3 : 5-di-iodo-4-(3′ : 5′-di-iodo-4′-hydroxyphenoxy) benzoic acid (IV.), since it is certain on chemical grounds that the two new iodine atoms must have entered the second benzene ring in the *ortho* positions to the free phenolic group. On methylation of this last mentioned acid there is obtained a product in every respect identical with the tetra-iodo acid prepared by methylation and reduction of thyroxine. Thus the identity of thyroxine was established beyond doubt as β-[3 : 5-di-iodo-4-(3′ : 5′-di-iodo-4′-hydroxyphenoxy) phenyl]-α-aminopropionic acid (V.).

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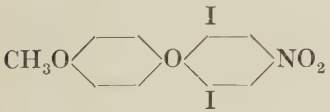

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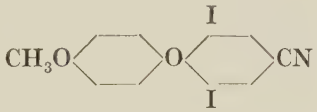

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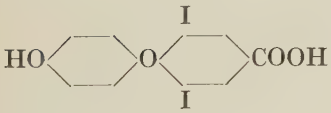

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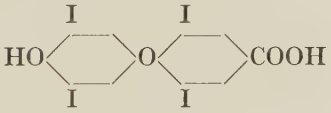

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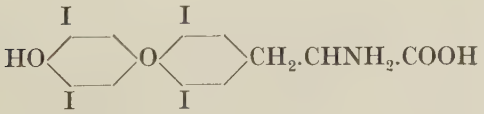
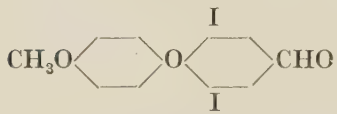


(Thyroxine)

The way was now clear for the attempted synthesis of thyroxine, and this was successfully accomplished and described by Harington and Barger in a remarkably short time after the elucidation of the structure by the methods already outlined. It is obvious that the main difficulty, and it is a formidable one, is the insertion of the four iodine atoms, but by starting with a compound already containing two iodine atoms these difficulties were lessened. Such a compound was available in the form of the nitrile (II.) already mentioned. This was converted to the aldehyde (VI.) by the reducing action of stannous chloride in anhydrous ether saturated with hydrogen chloride. The aldehyde was then condensed with hippuric acid and the resulting azlactone (VII.) on being subjected to acid hydrolysis yielded the corresponding α-benzoylaminocinnamic ester (VIII.). The latter was then boiled with hydriodic acid and red phosphorus whereby it underwent reduction and demethylation to form the compound (IX.), thyroxine minus two of its iodine atoms. The latter were inserted by treating compound IX. with iodine in ammoniacal solution, and the product then obtained proved to be identical in its chemical and physiological properties with thyroxine obtained from the thyroid gland. It should be remembered that this brief description makes no attempt to describe Harington's many painstaking investigations of other possible methods of synthesis of thyroxine, methods which yielded negative or poor results, and which were eventually discarded in favour of the series of reactions described above.



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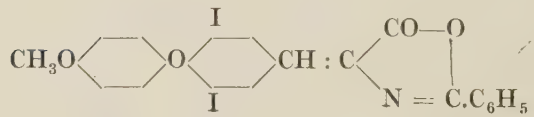

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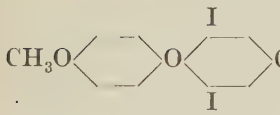

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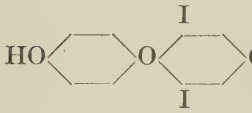


IX.

Synthetic thyroxine was subjected to physiological tests by being administered to patients known to be myxœdematous. In one case the basal metabolic rate was raised from −32 per cent. to −6 per cent. by the intravenous injection of a total of 18 mg. of the synthetic product over a period of eight days. Fifteen days after the first injection some ten pounds loss of weight had occurred, and the pulse rate had risen from 53 to 85. In a second case the injection of 14 mg. over a period of six days raised the basal metabolic rate from −46 per cent. to + 3 per cent. The temperature and pulse rate rose simultaneously and there was marked diuresis following the first two injections as a result of which a considerable proportion of excess weight was eliminated. Harington has recently been able to resolve thyroxine into its dextro and lævo rotatory forms, but so far only a slight difference in the physiological activity of the two stereoisomers has been observed.

The Tyrosinase-Tyrosine Reaction

Tyrosinase is an oxidising enzyme discovered by Bourquelot and Bertrand in 1896 and found widely distributed in vegetable tissues. Its presence has also been demonstrated in animal tissues. Its specific substrate is the amino acid tyrosine, which, in contact with the enzyme and in the presence of atmospheric oxygen, is oxidised ultimately to a black pigment, melanin. The latter name has been very loosely used both in chemistry and in biology. Practically any black, insoluble deposit found in tissues or in tissue products or formed during any biochemical opera-

tion has been called melanin, and the term has also been applied, but with less apparent justification, to the artificial products obtained by the prolonged action of oxidising agents, such as ferric chloride and hydrogen peroxide, on thiophen, benzene, phenols, cresols and other aromatic compounds, including tyrosine and its sulphonic acid. It is obvious that many of these artificial " melanins " do not contain nitrogen, and that they can bear no relationship to the natural melanin containing some 8·5 per cent. of nitrogen, obtained from tyrosine by the action of tyrosinase. Furthermore, some of these artificial " melanins " contain sulphur which is absent from natural " tyrosinase " melanin. It is true that many of the older analyses of melanin from natural sources record the presence of considerable percentages of sulphur, but many of these analyses have been carried out on obviously impure specimens of the pigment. Nevertheless it is possible that true, sulphur-containing melanins of natural origin may exist. Nothing is, however, known of their possible mode of formation.

The black, insoluble, naturally occurring pigments to which the name melanin is applied are found in the retinæ of mammals, in hair, feathers and horns, in the skin of negroid races, in melanotic tumours, in the layer of protective pigment (sun-burn) formed in the skin under the action of ultra-violet rays, in fish, *e.g.*, the cuttle-fish, *Sepia*, and in numerous other tissues of animal and plant origin. Whether or not these natural melanins are one and the same, and what their relationship is to the melanin which Raper and his colleagues have studied remains to be demonstrated. Meanwhile it is a reasonable assumption that this relationship is a close one.

One of the most convenient sources of tyrosinase is the mealworm, *Tenebrio molitor*, the larval form of the black beetle. Raper and Speakman have shown this tyrosinase to be identical with that prepared from the potato and from the fungus *Agaricus dryophilus*. From the mealworm it is

readily obtained by the following process. (Raper's modification of Gortner's process.)

100 gm. of mealworms are killed with chloroform water, washed and pounded with successive portions of chloroform water and the resulting fluid is strained through coarse muslin and filtered. The residue on the filter is washed with chloroform water containing a trace of acetic acid until the filtrate is colourless and gives only a slight turbidity with lead acetate and ammonia. This process usually occupies about ten days because the filtration is slow. The residue on the filter paper is now scraped off, ground in a mortar with small amounts of chloroform water and then transferred to a wide-mouthed stoppered bottle. A few cubic centimetres of chloroform are added and then ammonia until the reaction is slightly alkaline to litmus, with occasional shaking the enzyme gradually becomes very finely dispersed and constitutes a very active preparation of tyrosinase, which retains its activity for many weeks. If the solution be acidified slightly with acetic acid the enzyme is completely precipitated in an easily filterable condition.

When tyrosinase acts upon tyrosine in the presence of air or oxygen a series of colour changes is observed before finally the black, insoluble melanin separates from the solution. The most characteristic of these preliminary colour phenomena is the formation of a red pigment. Raper and Wormall in determining the limits of hydrogen ion concentration within which tyrosinase acts found that outside the limits of p_H 6·0 to 8·0 the action of the enzyme is very slow, and further that at p_H 6·0 tyrosine is converted very quickly to the red substance whilst the subsequent changes through reddish-brown to brown and black pigments are very slow. At p_H 8·0 the red colour also appears rapidly, but the subsequent darkening to brown and finally to black is also rapid. This suggested that the formation of the red substance is the first visible stage of the action of the enzyme and that the subsequent formation of melanin from it takes place more rapidly in the alkaline medium than at p_H 6·0. Advantage was taken of this observation to

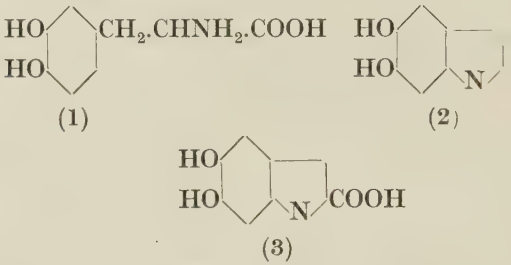
isolate the red intermediate product. By allowing the enzyme to act on tyrosine at p_H 6·0 and then, when the red colour was fully developed, by precipitating the enzyme from the sphere of action with colloidal ferric hydroxide the red substance is obtained free in solution. On boiling this solution the red colour rapidly disappears ; the same change also takes place, but much more slowly at room temperature *in vacuo* or in an atmosphere of hydrogen. On exposing the resulting colourless solution to air it darkens slowly and finally deposits melanin. At p_H 8·0 the darkening is very rapid. It would therefore appear that when the red substance is decolourised it gives rise to another product which is easily oxidised in the air especially in slightly alkaline solution. From these experiments Raper and Wormall concluded that the action of tyrosinase occurs in three distinct stages : (1) the oxidation of tyrosine to the red pigment, (2) the decolourisation of the red pigment with the formation of an easily oxidisable substance, (3) the oxidation of the colourless substance by molecular oxygen to melanin. Since stages (2) and (3) occur in the complete absence of the enzyme the latter is therefore only necessary for stage (1), the formation of the red substance.

Earlier workers, notably Chodat and Schweizer, had suggested that an essential step in the action of tyrosinase was a deaminising process, and Folpmers had isolated benzaldehyde from the products of action of tyrosinase on phenylaminoacetic acid. Happold and Raper were unable to detect any formation of ammonia or aldehyde when potato tyrosinase was allowed to act upon glycine, alanine, or phenylaminoacetic acid. Later, however, it was observed that ammonia formation did occur when *p*-cresol was present along with the amino acid. Chodat and Schweizer had in their experiments added this latter substance with the supposed object of preventing the interaction of the aldehyde and ammonia. Happold and Raper came to the conclusion that the deaminisation in the presence of *p*-cresol is not due

to the direct action of the enzyme, but is caused by the preliminary oxidation of the *p*-cresol with the formation of an *ortho*-quinone derivative which then attacks the amino group of the amino acid. In the presence of phenol and catechol deaminisation also occurs but not in the presence of resorcinol or quinol. When it was shown that the direct addition of *o*-benzoquinone to amino acid solutions, in the absence of tyrosinase, caused deaminisation, it appeared probable that *o*-quinones generally were responsible for the phenomenon, and that those substituted benzenes capable of being oxidised to *o*-quinones would produce deaminisation in the presence of the enzyme. Thus the failure of quinol and resorcinol to bring about the deaminisation was readily explained, and it was further shown that *p*-benzoquinone, unlike the *ortho* isomer, had no direct action on amino acids. It was later shown in Raper's laboratory that tyrosinase acting directly on both phenol and *p*-cresol produces *o*-benzoquinone and 3 : 4 toluquinone respectively. It now seemed likely that since tyrosine contains a phenolic hydroxyl group these observations might have an important bearing on the mechanism of action of tyrosinase, and that an *ortho*-quinone derivative of tyrosine might play the part of an intermediate substance, since the production of *ortho*-quinones from suitable phenolic bodies is a specific action of tyrosinase.

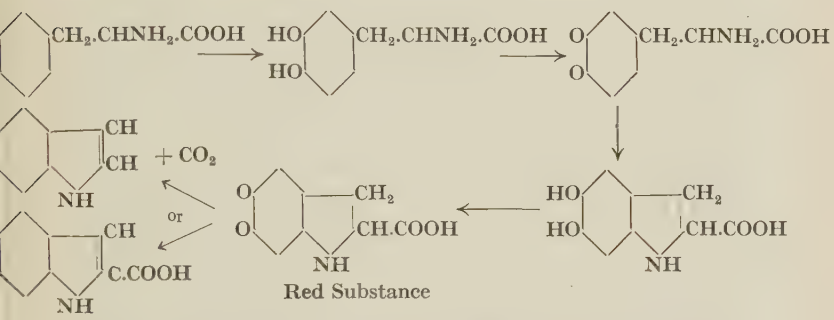
In order to elucidate this point mealworm tyrosinase was allowed to act on tyrosine at p_H 6·0 until a maximum development of the red colour has occurred, the enzyme was then precipitated by the addition of acetic acid, and there was obtained a solution containing unchanged tyrosine along with the products arising from it, one of these being the red substance. The solution obtained in this way was then allowed to decolourise either by standing *in vacuo* or by adding a small amount of sulphurous acid, and from the decolourised solution three substances were isolated, namely, (1) *l*-3 : 4-dihydroxyphenylalanine, (2) 5 : 6-dihy-

droxyindole, (3) 5 : 6-dihydroxyindole-2-carboxylic acid, the formulæ of which are given below :



3 : 4-Dihydroxyphenylalanine was obtained in the crystalline *lævo*-rotatory form by precipitating the still acid, decolourised solution with lead acetate and allowing the substances (2) and (3) to oxidise to melanin. The dihydroxyphenylalanine is left in solution and is precipitated from the filtrate as the lead compound on making the solution slightly alkaline with ammonia. From the lead compound the free acid is liberated by means of hydrogen sulphide. 5 : 6-Dihydroxyindole is obtained by allowing the red substance to decolourise in an atmosphere of hydrogen, whilst its 2-carboxylic acid is obtained in the presence of sulphurous acid. Since both these compounds are, however, very susceptible to oxidation special precautions must be taken to exclude air and to render them more stable. This is achieved by subjecting the decolourised solution kept in an atmosphere of hydrogen to the methylating action of dimethyl sulphate and sodium hydroxide. The compounds are then isolated in the form of their stable dimethyl derivatives. These two indole derivatives were unknown until prepared by the above essentially biological method, but Oxford and Raper subsequently prepared them by purely synthetic chemical means, and showed the products to be identical with those obtained with the help of tyrosinase, thus verifying the constitutions assigned to the naturally formed indoles.

Thus it appears highly probable that the first product of the action of tyrosinase upon tyrosine is 3 : 4-dihydroxyphenylalanine, and this is strongly supported by the following facts. 3 : 4-Dihydroxyphenylalanine is readily oxidised by tyrosinase and much more rapidly than is tyrosine itself ; it gives rise to a red substance in the initial stages of the reaction ; employing the conditions leading to the isolation of 5 : 6-dimethoxyindole and its 2-carboxylic acid from tyrosine, the same two products are isolated from dihydroxyphenylalanine when it is acted upon by tyrosinase. In explaining the subsequent formation of indole compounds Raper makes use of the known property of tyrosinase to form *ortho*-quinones, a property which we have already discussed. It is suggested that the following is the series of reactions which takes place in the tyrosinase-tyrosine reaction preliminary to the formation of melanin :



The first product is 3 : 4-dihydroxyphenylalanine, and this is next oxidised to the corresponding quinone, which then undergoes an intramolecular change (analogous to the formation of an anilino-quinone), and the nitrogen of the side chain becomes attached to position 6 of the benzene ring. The 5 : 6-dihydroxydihydroindole-2-carboxylic acid is then oxidised to its corresponding quinone which is believed to be the red substance, the first visible product of the enzyme action. The enzyme is not necessary for

the further stages of the reaction. When the red substance becomes decolourised it gives rise to 5 : 6-dihydroxyindole or its 2-carboxylic acid. It is obvious that the formation of the latter compound is simply an auto-reduction of the red substance, two hydrogen atoms migrating from the pyrrole ring and becoming attached to the two *ortho*-quinone oxygen atoms. Neither 5 : 6-dihydroxyindole nor its 2-carboxylic acid have been prepared synthetically but it is certain that they would both be extremely easily oxidised in contact with air and these two substances are regarded as the immediate precursors of melanin. Raper explains the more rapid formation of melanin at p_H 8·0 than at p_H 6·0 by the more rapid change of the 5 : 6 quinone of dihydroindole carboxylic acid into the corresponding indole derivatives on the alkaline side of the neutral point, and by the greater ease of oxidation of these substances at an alkaline p_H.

The constitution of melanin itself remains undetermined. It contains 8·65 per cent. of nitrogen according to the Dumas method and 8·40 per cent. by Kjeldahl's method. Raper points out that if two atoms of hydrogen be lost and one atom of oxygen be taken up in the formation of melanin from dihydroxyindole, then the product $(C_8H_5O_3N)_n$ would contain 8·59 per cent. of nitrogen, and if oxygen alone were taken up the product would have the composition $(C_8H_7O_3N)_n$ and would contain 8·49 per cent. of nitrogen. Union of many molecules of indole certainly takes place since melanin is a colloidal, amorphous substance. It also seems probable that because of its black colour it contains quinonoid arrangements within its complex molecule.

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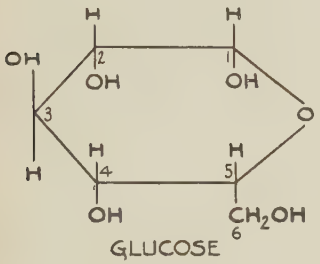
CHAPTER V

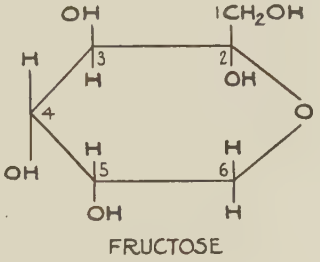
THE CARBOHYDRATES

The average mixed diet of man consists of some 50 to 60 per cent. of starches and sugars, and it is estimated that of the proteins some 60 per cent., and of the fats approximately 10 per cent. (the glycerol), are transformed into sugar in the course of normal metabolism. It is therefore not surprising to find that the bulk of man's energy requirements are met by the oxidation of carbohydrate. This oxidation occurs to a great extent in muscle tissues, where it is probable that the only sugar directly oxidised is *d*-glucose. The metabolism of the carbohydrates is therefore practically that of glucose, and the ease and extent of transformation of the other sugars into glucose is a measure of the extent to which they are likely to be utilised in the course of normal metabolism. Thus sugars which most closely resemble glucose in their stereochemical configuration are more readily utilised by the mammalian organism than are sugars which depart more markedly from this configuration. In the former class we have fructose, the ketose analogue of glucose, and mannose, the epimer of glucose, in which the stereochemical configuration of the $\rangle$CHOH group adjacent to the terminal carbon atom (No. 1) is reversed as shown in the accompanying formulæ (p. 89). These embody the new six-membered ring structure now ascribed to the normal sugars (*see* p. 100). The five carbon atoms and the bridge oxygen atom are pictured in one plane (perpendicular to the plane of the page), whilst the individual stereochemical configuration of

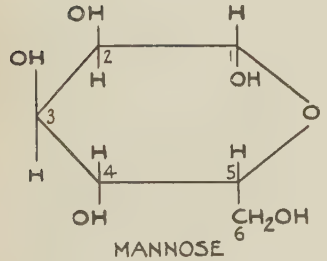
the various sugars is shown by the position of the substituent hydrogen atoms and hydroxyl groups above or below the plane of the six ring atoms. This method of formulation is



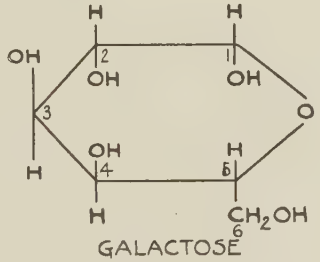
GLUCOSE



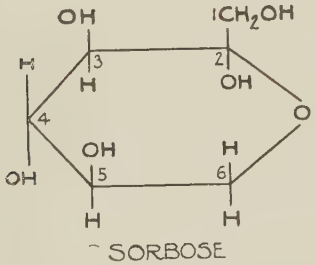
FRUCTOSE



MANNOSE



GALACTOSE

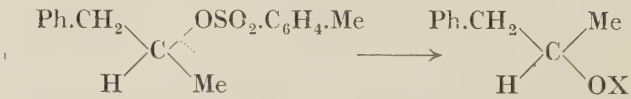


SORBOSE

to be recommended since it serves to emphasise the structural similarities between the carbohydrates and other cyclic compounds.

In the cases of galactose and sorbose we have sugars which

differ from glucose in a stereochemical sense more markedly than do mannose and fructose. The reversal of the stereochemical configuration of carbon atoms 4 and 5 is a much more difficult chemical and biochemical problem than is the corresponding reversal of carbon atom 2, the proximity of the latter to the reducing carbon atom (No. 1) doubtless being the factor determining the difference in the aldoses. Thus it is doubtful if galactose is utilised at all in the sense of being oxidised, although it is one of the constituent hexoses of the mammalian milk-sugar lactose and is present in the cerebrosides, which occur in large amount in brain tissue. It is nevertheless probable that the galactose in these sources is formed at least in part by a synthetic process, and is not derived wholly from preformed ingested galactose. It is further stated that lactose, when fed to diabetics, may be recovered in the urine as 2 molecules of *d*-glucose for each molecule of lactose fed. These observations would seem to indicate that the tissues can under certain conditions transform glucose into galactose, and galactose into glucose. Such a change is very interesting from a stereochemical point of view, and Professor R. Robinson, F.R.S., has recently made a very helpful suggestion as to the possible mechanism of this and similar changes. Reference has already been made to this suggestion in relation to the nature of the sugar of plant nucleic acid (p. 53). Recent studies of the Walden inversion have shown that when asymmetric optically active sulphonic acid derivatives are hydrolysed fission is normally followed by optical inversion. Thus on acetolysis of *d*-benzyl methylcarbinyl *p*-toluene sulphonate inversion is observed, the process being pictured thus :—



Sulphate and phosphate substituent groups, having a similar high affinity for oxygen, would behave in the same

way as aryl-sulphonyl groups in bringing about inversion. Considering the widespread occurrence of sugar phosphates in nature and their participation in numerous important biochemical processes, Professor Robinson suggests that the reaction glucose $\rightleftharpoons$ galactose and similar reactions are normally brought about by the formation and subsequent hydrolysis, with optical inversion, of a hexose-4-phosphate. The result of such a process would obviously be the reversal of the stereochemical configuration of the carbon atom to which the phosphate group was attached, thus :—

2	H	OH		H	OH
3	HO	H	⟶	HO	H
4	H	OH	⟵	HO	H
5	H	OH		H	OH
	d-glucose			*d*-galactose	

Helpful as these suggestions are, there are nevertheless considerable limitations to the capacity of living tissues for interchanging carbohydrates. Thus Laquer and Meyer have demonstrated in their studies on the metabolism of carbohydrate in muscle that, whilst lactic acid is readily formed from glycogen, starch, glucose, fructose, and mannose, it is not formed in significant amounts from galactose and sorbose. The table on p. 92, taken from their paper, shows these results together with some others of interest.

The Pentoses

The pentoses and methyl pentoses do not appear to be metabolised in the majority of mammals in appreciable amounts. In the herbivorous animals, however, which ingest a large amount of pentosans (pentose polysaccharides) in their foodstuff, a considerable absorption of pentose takes place. The subsequent fate of this pentose is obscure. It has been claimed that arabinose, xylose, and rhamnose, when fed to starving rabbits, are transformed into glycogen.

TABLE VIII

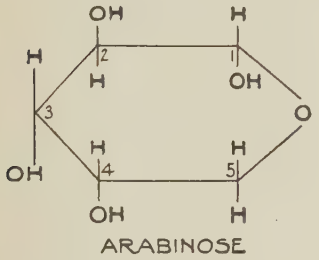
Formation of Lactic Acid from various Carbohydrates by a Pulp of Frog Skeletal Muscle at 30°. Sugar Concentration 2 per cent. p_H 7·3.

Carbohydrate Added.	No. of Expts.	Excess Formation of Lactic Acid over Controls. Gm. per 100 gm. muscle.		
		Lowest.	Highest.	Average.
Glucose .	25	+ 0·008	+ 0·828	+ 0·395
Fructose .	6	+ 0·322	+ 0·647	+ 0·484
Mannose .	4	+ 0·173	+ 0·695	+ 0·327
Galactose .	4	— 0·001	+ 0·030	+ 0·014
Sorbose .	2	— 0·013	+ 0·062	+ 0·024
Sucrose . .	2	— 0·030	+ 0·064	+ 0·017
Maltose .	8	— 0·055	+ 0·108	+ 0·026
Diamylose .	6	— 0·068	+ 0·038	— 0·004
Tetramylose .	3	+ 0·006	+ 0·077	+ 0·035
Inulin . .	2	— 0·095	+ 0·031	+ 0·032
Starch . .	4	+ 0·115	+ 0·772	+ 0·466
Glycogen .	23	+ 0·350	+ 1·015	+ 0·707

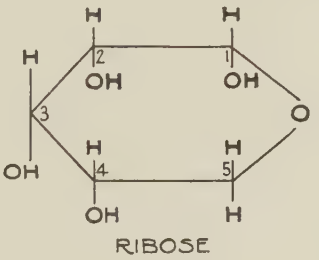
Substantiation of these results in recent years appears to be lacking, and it is obvious from the formulæ on p. 93 that such a transformation in the instances of xylose and rhamnose involves a considerable stereochemical rearrangement of the molecule. If such glycogen formation does take place, one would therefore suggest that it is by way of disintegration products of two or three carbon atoms rather than by a direct conversion of the pentose to hexose. It has also been stated that diabetics excrete glucose when pentoses are administered to them, but in relation to these claims it must be borne in mind that the administration of large quantities of pentose to diabetics has a stimulating effect on their protein catabolism, and the supposed formation of glucose might be due to this indirect process.

In general, it may be said that the pentoses are far less readily utilised in the animal organism than are the hexoses,

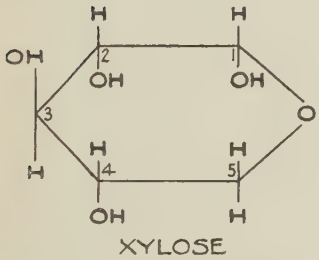
and after administration to the animal they are largely excreted unchanged. One might have anticipated that the conversion of a pentose such as arabinose into a hexose such as glucose would be a comparatively simple biological problem. The stereochemical configuration of the two sugars is very similar, and the change is comparatively easy to achieve *in vitro*. But the form of arabinose which does



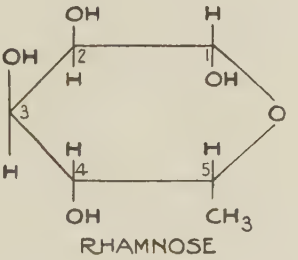
ARABINOSE



RIBOSE



XYLOSE



RHAMNOSE

occur in nature is *dextro*-rotatory, whereas the arabinose derived from glucose of the natural or *dextro*-rotatory variety, by a simple degradation process such as Weerman's, proves to be *lævo*-rotatory. It is, therefore, the optical enantiomorph of the natural form of arabinose. This fact serves to emphasise the unexpected difficulties one encounters in attempting to view the biological origin and behaviour of the pentoses and hexoses from a common standpoint. On the other hand, one feels fairly confident in the prediction

that in the plant the pentose is derived from a hexose by some process of degradation, and Professor Haworth has recently pointed out that, whilst direct degradation of *d*-glucose would give rise to *d*-arabinose, a form which is not found in nature, the decarboxylation of *d*-galacturonic acid would give rise to the *l*-form, that is the natural form, of this pentose. Reference to the cyclic formulæ already given will demonstrate how the two enantiomorphs would be formed by these different processes. The frequent occurrence of pentoses and " uronic " acids in the same natural source is certainly suggestive in this connection. In the animal, if the pentoses are metabolised at all, it would seem to be by methods quite different from those which apply to the hexoses. It is indeed surprising that the only pentose normally occurring in animal tissues is *d*-ribose, which is found in the so-called β-nucleo-proteins, already referred to in Chapter III. The configuration of ribose relates it to the rare hexose sugars *d*-allose and *d*-altrose, which so far have only been obtained by artificial means and are not known to occur in nature. They were obtained by Levene and Jacobs on applying Kiliani's cyanohydrin synthesis to ribose. (*See also* p. 53.)

In the rare condition known as pentosuria there is a continued excretion of a pentose in the urine. This pathological state has not been correlated with any other significant metabolic disturbance, and is not associated with diabetes, although the condition has usually been brought to light through a faulty diagnosis of diabetes. The pentose has not yet been satisfactorily identified. The following list summarises the opinions of various investigators of this question :—

Salkowski (1899) . . .	*dl*-arabinose.
Neuberg (1900) . . .	*dl*-arabinose.
Blumenthal (1903) . .	*d*-rotatory pentose.
Adler (1905) . . .	*l*-rotatory pentose.
Luzatto (1905) . . .	*l*-arabinose.
Blum (1906) . . .	*l*-rotatory pentose.
Rosenfeld (1906) . . .	*d*-rotatory pentose.
Schuler (1910) . . .	*d*-rotatory pentose.

Elliot and Raper (1912)	*dl*-ribose.
Aron (1913)	*dl*-arabinose.
Zerner and Waltuch (1913)	*d*-xyloketose.
Levene and La Forge (1913)	*l*-ribose.
Levene and La Forge (1914)	xyloketose.
Hillier (1917)	*d*-xyloketose.
Wrzesnewski (1922)	*dl*-arabinose.

It would seem from the above list that it is by no means improbable that different types of pentosuria exist.

The Relation of the Structure of Carbohydrates to their Biochemical Transformations

The bulk of man's carbohydrate intake is in the form of starch, the amount of free mono- and di-saccharides ingested in foodstuffs being relatively insignificant. Before absorption starch is completely hydrolysed to glucose by the successive actions of amylases (diastases) and maltase, and that portion of the glucose which is not immediately utilised is stored as glycogen, principally in the liver and muscles. The series of reactions representing this conversion, namely,

$$\text{Starch} \longrightarrow \text{Glucose} \rightleftarrows \text{Glycogen},$$

which plays an important part in the utilisation of carbohydrates in the organism, raises the question of the relationship of starch to the animal polysaccharide glycogen.

Recent investigations of the constitution of starch indicate that it is probably a polymerised anhydro-tri- or hexasaccharide. The view that the essential unit of starch is an anhydro-trisaccharide or triamylose, in which two of the anhydro linkings are of the same type and the third of another type, is favoured by Irvine and by Pringsheim. Ling and Nanji, taking cognisance of the existence within the starch granule of amylopectin and polymerised amylose, two distinct polysaccharides occurring in the ratio 1 : 2, question certain of Irvine's conclusions. Amylopectin, which contains a small amount of probably combined phosphoric acid, forms the outer layers of the starch granules, whilst amylose is found in their interior. Both polysaccharides give rise to

the same chief constituent, glucose, on acid hydrolysis, but they reveal differences when they are subjected to enzymic hydrolysis. Thus, when starch paste is treated at 50° with precipitated barley diastase which has been dried by treatment with strong alcohol, it is converted practically quantitatively to maltose (glucose α-glucoside) in about twelve hours, whilst the amylopectin is merely depolymerised to an anhydro-hexasaccharide. The maltose may be removed by dialysis and the unchanged amylopectin then precipitated by alcohol. Amylopectin on suitable enzymic hydrolysis yields maltose and *iso*-maltose (glucose β-glucoside). Ling and

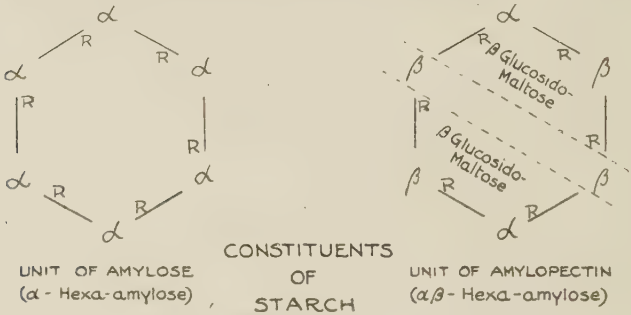
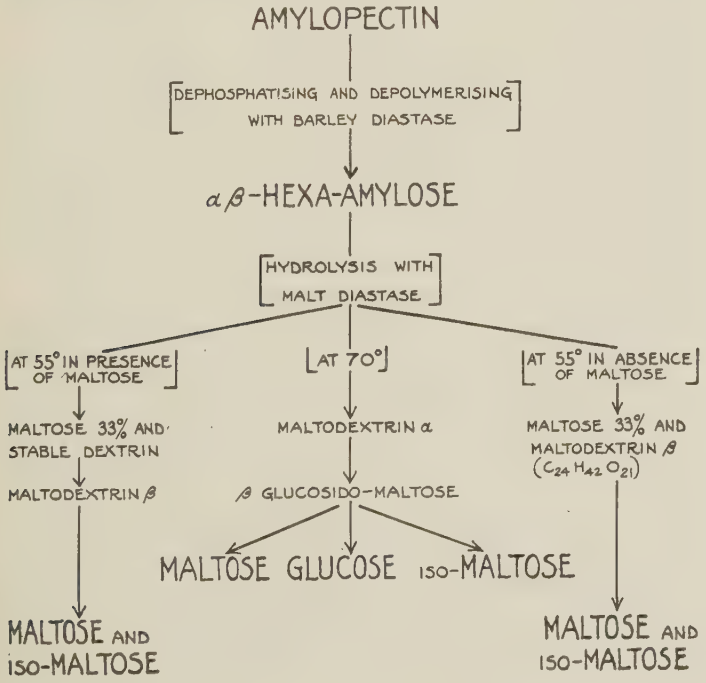


FIG. 9.

Nanji suggest that the polymerised unit of the amylose of starch is α-hexa-amylose, *i.e.*, an anhydro-hexasaccharide in which the glucosidic linkings are of the α type. This hypothesis readily explains the occurrence of tetra- and di-amyloses in starch hydrolysates by a process of further depolymerisation. For amylopectin the constitution suggested by Ling and Nanji for the polymerised unit is an $\alpha\beta$-hexa-amylose, in which two carbinol hydroxyl groups adjacent to β linkings are esterified with phosphoric acid. The necessity for supposing the presence of the β linking depends on the production of *iso*-maltose on hydrolysis. Four of the linkings between the constituent hexose units are supposed to be of this type, whilst the other two are presumed to be of the α type. This latter hypothesis con-

cerning the number of each type of linking accommodates the fact that, by the action of malt diastase at 70° on amylopectin, the sole product obtained under certain conditions is the trisaccharide β-glucosido-maltose. These suggested structures of the units of amylose and amylopectin of starch are shown in the diagrams in Fig. 9. The lines forming the sides of the hexagons represent the individual hexoses, the letter R indicating the reducing end of the molecule. The type of linkage between each hexose unit is shown by the letters α or β. The dotted lines running across the figure representing the amylopectin unit show how scission of this unit may occur with the production of β-glucosido-maltose only.

The following scheme is given by Ling and Nanji as representing the course of enzymic hydrolysis of amylopectin under various conditions :—

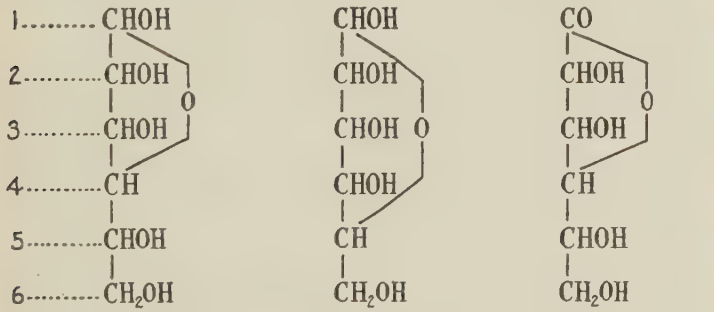


In various starches, notably those of barley, wheat, rice, sago, maize, and tapioca, Schryver has discovered a hemicellulose in which a certain amount of silica is present in organic combination. This discovery further complicates the chemistry of starch. The hemicellulose gives a blue iodine reaction, and may therefore be confused with starch. It is not, however, acted on by the diastase of ungerminated barley even on prolonged incubation at 50°, whereas the other two constituents of starch, the amylose and the amylopectin, are hydrolysed or depolymerised by this enzyme under these conditions. The hemicellulose is hydrolysed by the diastase of germinated barley.

The foregoing observations serve to show how complex the biochemical reactions of starch may be, and there is no reason to suppose that the behaviour of glycogen in the animal body is materially simpler, although up to the present time the detailed structure of glycogen has not been so extensively investigated as has that of starch. Glycogen closely resembles starch in being a polysaccharide of the formula $(C_6H_{10}O_5)_n$, in which each C_6 unit carries three hydroxyl groups. Macbeth and Mackay have succeeded in preparing a methylo-glycogen in which the methoxyl content is within 5 per cent. of the theoretical value for the completely methylated substance. The difficulty in effecting complete substitution of the hydroxyls by methoxyl is probably physical. On hydrolysis of this partly methylated glycogen, one of the products obtained proved to be a trimethyl glucose identical with that obtained from methylated cellulose, starch, lactose, and cellobiose, a disaccharide prepared from cellulose. The residue consisted chiefly of a dimethyl glucose similar to that obtained from starch by Irvine and his co-workers. There are, therefore, good grounds for believing that starch and glycogen are built up on closely similar lines. There is as yet no evidence that the polysaccharide glycogen is related to or built up from glucose units of the abnormal or reactive type, the so-called γ type.

In recent years a good deal of speculation has been devoted to the possible *rôle* of reactive sugars in biochemistry, and a short *résumé* of the position is given here.

Reactive or γ sugars were first discovered in the form of their glucosidic derivatives by Fischer, and shortly afterwards by Irvine and his co-workers, in 1914. The difference between these reactive sugars and the normal sugars is ascribed to a displacement of the oxygen bridge, which links the terminal reducing carbon atom to another carbon atom, from its position in the normal stable sugars. Up till very recently it has been assumed that the oxygen bridge present in the stable sugars was of the butylene oxidic type, that is, that it linked carbon atoms 1 and 4 as shown below. Definite



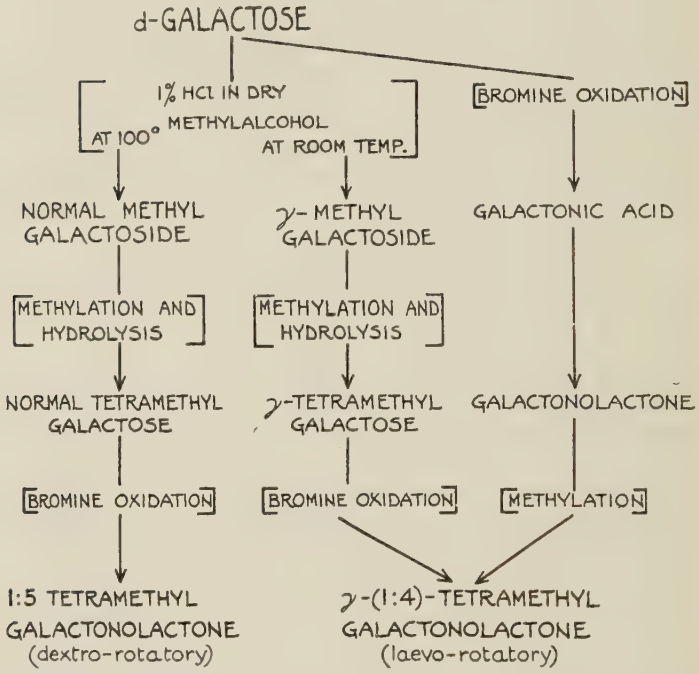
BUTYLENE OXIDIC SUGAR

AMYLENE OXIDIC SUGAR

BUTYLENE OXIDIC MONO-CARBOXYLIC ACID LACTONE

evidence in favour of this type of linking was lacking, and the assumption was made because it was thought to be a very stable atomic grouping, and because Hudson had brought forward unquestioned evidence that the lactones of the monocarboxylic acids derived from the sugars on oxidation existed in the 1 : 4 form, *i.e.*, as γ-lactones. As a result of recent work carried out by Hirst, Haworth, Levene, the author, and their co-workers, it has been possible to demonstrate that in all the aldo-hexoses and aldo-pentoses so far

examined—and these include glucose, mannose, galactose, fructose, xylose, arabinose, and rhamnose—the stable structure is that in which the oxygen bridge links carbon atoms 1 and 5, so that the butylene oxidic structure hitherto assigned to these sugars must now be replaced by an amylene oxidic or six-membered ring structure as shown in the



formulæ given on pp. 89 and 93. It has further been shown in these recent studies that the γ or reactive form of the aldoses is none other than the butylene oxidic form which has for years been ascribed to the stable sugar. We see, therefore, that the type of oxygen bridge which is most stable in the acid lactones, when present in the sugars, confers on them highly reactive properties, and the original

selection of the title γ-sugar for the reactive type has been fortuitously justified by the fact that this type of sugar gives rise on oxidation to a γ-lactone without change in the position of its oxygen bridge. As illustrative of some of the details of this recent work, a scheme summarising the combined results of Haworth and of the author on galactose is appended (p. 100). The author and Mr. Humphreys have recently published a similar but more complete scheme of the transformations undergone by the pentose arabinose.

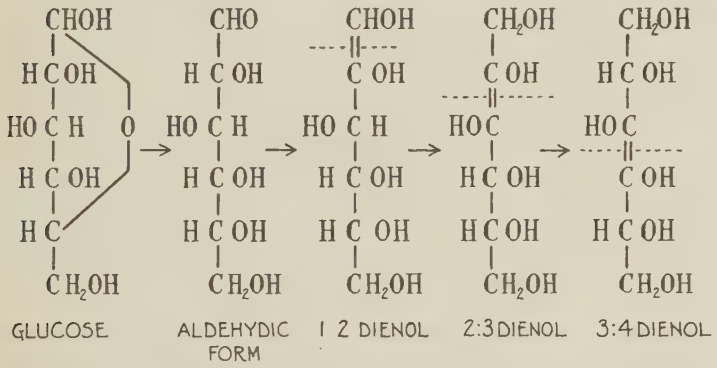
If, as shown in the above series of reactions, the position of the oxygen bridge is prevented from altering by substituting all the other hydroxyl groups by methoxyl, one obtains from the normal series of derivatives a *dextro*-rotatory lactone, whereas in the γ series, and on direct oxidation of the free sugar and on subsequent methylation, identical *lævo*-rotatory lactones are obtained. The opposition in the direction of the rotation shown by the two lactones is in accord with the fact that in galactose the hydroxyl groups attached to the carbon atoms 4 and 5 lie on opposite sides of the carbon chain. In the cases of glucose and mannose, since the corresponding hydroxyl groups lie on the same sides of the carbon chain, one expects the lactones prepared in the same way as is described above to have the same direction of rotation, but to differ quantitatively in this constant, and such is in fact observed.

The ease with which a sugar in which the oxygen bridge is displaced from its normal position undergoes oxidation has exercised a strong fascination on biochemists. The hypothesis that the reactive form of glucose may play an important part in the metabolism of carbohydrates is certainly attractive, but it must be confessed that no convincing evidence is forthcoming in support of this supposition, nor, on the other hand, is the hypothesis disproved. No single instance of an aldohexose occurring in nature in its reactive form, either in the combined or in the free state, has yet been brought to light. On the other hand, the ketohexose fructose, as it is found in

the polysaccharide inulin, in the disaccharide sucrose, and in the trisaccharides raffinose, gentianose, and melezitose, is much more reactive in this form than it is in stable crystalline fructose. It is now known that whereas the latter has the normal amylene oxidic structure, the combined form of this ketohexose is butylene oxidic.

From polarimetric observation of fluids of physiological origin, such as deproteinised blood filtrates, which contain carbohydrate amongst numerous other components, it has been argued that the sugar present is not the normal equilibrium mixture of the α- and β-stereoisomerides of stable glucose. Thus it is found that the optical rotatory power of such fluids is frequently below that required for a solution containing the equivalent amount of glucose as calculated from the copper reducing power. Moreover, the observed rotation frequently changes with lapse of time, and a closer correspondence between the two values may be attained. Sometimes the reverse is the case. Deductions from such observations must be accepted with great caution, since one is dealing with solutions of sugar contaminated with many other substances of which the influence on the rotation of the sugar is unknown, and which may form more or less stable compounds with the sugar. The inference that such disparities between polarimetric and copper-reducing values for the sugar present in these solutions indicate the presence of reactive forms seems unwarranted. Similar abnormal polarimetric observations have been obtained by Stasiak in working with blood serum, and have been imitated *in vitro* with pure mixed solutions of glucose and globulin, and of glucose and cholesterol. The type of complication that may arise, even in blood filtrates from which the protein has been removed by the usual methods, is indicated by the presence in pig's blood of an adenine pentose nucleotide (Chapter III.), and the interesting discovery by Benedict and his associates of a uric acid-*d*-ribose (*cf.* p. 53) compound in the blood of various animals, more particularly in human and in ox blood.

The suggestion of Neuberg that in pentosuria the excreted pentose is combined at least in part with urea is of interest in a similar direction. Some years ago Morrell and Bellars reported the formation of definite compounds of hexoses and guanidine, and more recently Witzemann has investigated the influence of guanidine on the chemical and polarimetric behaviour of glucose. Its general effect is to produce a lowering of the rotation of the sugar in a manner similar to that of dilute alkali, so that the amount of sugar as calculated from the rotation of the solution is below that determined by copper reduction methods. Compounds of which guani-



dine forms a part are known to be present in blood, where they may exert an action similar to that obtained *in vitro* with free guanidine. It may be remarked in passing that the use of " Synthalin," a synthetic di-guanidine derivative, as a substitute for insulin in diabetes is a practical illustration of the point in question. In any case, the known instances of associations between sugar and other components of blood are sufficiently suggestive of other possible combinations, the presence of which would certainly lead to discrepancies between sugar determined by the two methods which we have mentioned. The further fact that compounds of the type of Benedict's uric acid-pentose derivative

would yield on hydrolysis a sugar distinct from glucose provides another possible complication.

When present in an alkaline solution, the reactivity of a sugar is greatly increased. It has been known since the classical work of Lobry de Bruyn and van Ekenstein, and the more recent work of Nef, that in the presence of alkali of varying strength glucose undergoes an extensive series of transformations, in the course of which other hexoses, including *d*-mannose and *d*-fructose, are formed, and these, together with the unchanged glucose, then undergo further degradation. If oxygen be admitted, oxidation occurs much more readily in such alkaline solutions than in neutral or acid solutions. Nef ascribes these results to the initial formation from the mixture of hexoses of 1 : 2, 2 : 3, and 3 : 4 di-enol forms, the open-chain aldehydic isomer probably being an intermediate product, as shown on p. 103.

In the presence of strong alkali these forms are extensively disrupted at the points indicated by the dotted lines, and numerous degradation products, such as formaldehyde and aldopentose, dioses and aldotetroses, and trioses are formed. The trioses are quantitatively the most important of these products, and as we shall see, such three-carbon compounds are the first recognisable degradation products which are known to occur when glucose is metabolised *in vivo*. The best known of these products formed in the living tissues is lactic acid. This acid is also found when glucose is degraded *in vitro* in the presence of alkali, if oxygen is excluded from the system.

Another set of conditions under which a hexose is readily oxidised by molecular oxygen is brought about by the presence of the phosphates of the alkali metals. Thus Warburg and Yabusoe have shown that fructose, but not glucose, is oxidised in this way with the formation of carbon dioxide. As we shall see in a later chapter, the metabolic transformations which glucose undergoes in yeast fermentation and in muscle contraction are characterised by the

formation, as intermediate products, of hexose phosphates, probably compounds of fructose. It appears, however, from Meyerhof's and from Neuberg's observations that the oxidation of fructose in the presence of phosphate is a catalytic process and does not involve the formation of hexose phosphate as an intermediate step. Nevertheless, this serves as a suggestive analogy to the biochemical process in which phosphate plays a fundamental intermediate *rôle* between the sugar and alcohol and carbon dioxide in the instance of yeast fermentation, and between the sugar and lactic acid in the muscle process.

In the muscles, where the bulk of carbohydrate oxidation occurs, lactic acid is quantitatively the most important and most easily demonstrated known intermediary. But recent work makes it probable that the lactic acid is not further oxidised, but is retransformed through hexose phosphate and glucose to glycogen, oxygen being necessary for the process. If the oxygen is deficient or absent, the muscle "fatigues" through the accumulation of the lactic acid under the anaerobic conditions. It is during the phase of liberation of lactic acid that the contraction of the muscle occurs, and during the reversal or "recovery" phase a further supply of sugar is oxidised to supply the necessary energy for the building-up process. It is this latter portion of the total sugar only which is directly oxidised, and from it is formed the carbon dioxide which is the final product of carbohydrate metabolism. Although the production of lactic acid is a fundamental part of carbohydrate metabolism and is intimately associated with the contraction of muscle, we cannot from this point of view regard it as an intermediate in the direct oxidation of glucose. It is, of course, possible that the glucose which is directly oxidised is first converted, like the rest of the glucose, to lactic acid, but there is no evidence that this is actually the case. Hartree and Hill have shown in a study of the heat evolved during the various phases of muscular contraction that only some 1/4·7 to 1/6

of the total lactic acid produced during the contraction of the muscle (or the equivalent of this amount of acid in the form of carbohydrate) is directly oxidised after the contraction.

Meyerhof, Lohmann, and Meier have recently shown, in an instructive series of experiments in which they investigated the synthesis of carbohydrate from lactic acid in the muscle, that added lactic acid participates in the " recovery " process in the same quantitative way as the acid formed during contraction. They have shown that the glycogen content of frog's muscle increases when it is perfused with oxygenated Ringer solution containing lactic acid, but they have obtained much more decisive results by the simple expedient of immersing isolated muscles, such as the sartorius or gastrocnemius, in the lactic acid-Ringer solution. They recorded the oxygen uptake of the muscle during the process of synthesis, and have been able to demonstrate that synthesis and oxygen consumption run parallel to one another. If the ratio between the total amount of lactic acid which disappears and the equivalent amount of lactic acid (or sugar) which is oxidised, as judged by the oxygen consumption, be determined, one obtains what is called the *oxidation quotient*, as shown in the table on p. 107 taken from the paper of Meyerhof and his collaborators. In all the cases recorded the corresponding muscle from the opposite leg was used in a control experiment in which no lactic acid was added.

In the last two experiments quoted, Nos. 6 and 7, the glycogen alone was determined. The results are very close to those obtained in the other experiments, in which the total carbohydrate as well as the glycogen was determined. We may infer that the lactic acid which is used in the synthetical process is built up not merely into glucose, but into the polysaccharide glycogen. It will be seen that the average value of the oxidation quotient is 4·33, which is in good agreement with the same quotient as determined (1) during recovery of the muscle after contraction, (2) after a period of rest under anaerobic conditions, (3) during rest under

TABLE IX

Carbohydrate Synthesis from Lactic Acid added to Muscle.

A. *Oxygen Consumption.*

Expt. No.	Weight of Muscle.	Duration of Expt.		Cms. O_2 consumed in :—		Extra Oxygen Consumption in Mgms.
				Control.	Lactic Acid Muscle.	
	gm.	hrs.	mins.			
1	1·30	21	0	943	1,385	0·63
2	1·30	18	30	783	1,025	0·345
3	2·08	18	20	1,212	1,686	0·678
4	1·49	6	30	514	1,200	0·98
5	1·28	16	0	515	977	0·661
6	1·05	15	30	479	628	0·213
7	1·50	15	30	786	1,133	0·497

B. *Carbohydrate Synthesis.*

Expt. No.	Mgm. Carbohydrate in :—		Per cent. Carbohydrate in :—		Carbohydrate Synthesised.		Equivalent of Lactic Acid Oxidised. Mgms.	Oxidation Quotient.
	Control.	Lactic Acid Muscle.	Control.	Lactic Acid Muscle.	Per cent.	Mgm.		
1	15·1	17·2	1·16	1·32	0·21	2·7	0·59	4·7
2	15·2	17·2	1·165	1·32	0·18	2·35	0·323	7·2
3	19·7	21·3	0·94	1·03	0·12	2·23	0·633	3·5
4	16·4	17·5	1·10	1·17	0·13	2·02	0·92	2·2
5	20·3	22·0	1·585	1·72	0·185	2·32	0·62	3·75
6*	7·4	8·35	0·71	0·80	0·11	1·15	0·20	5·7
7*	13·1	14·15	0·87	0·94	0·105	1·52	0·465	3·25
					Average oxidation quotient .			4·33

* Glycogen only determined.

aerobic conditions, (4) in minced muscle under aerobic and anaerobic conditions, and (5) in the fatigued entire animal. In all these cases values between 3 and 5·5 are obtained. In the experiments of Hartree and Hill already mentioned the

oxidation quotient which would correspond to the observed ratio between total lactic acid removed and equivalent of lactic acid oxidised would lie between 4·7 and 6, the average value being about 5. There is therefore excellent correspondence between the physical methods of the latter investigators and the chemical methods employed by Meyerhof.

We have already stated that the bulk of carbohydrate oxidation takes place in the muscles, and it would appear that carbohydrate is oxidised here in preference to other foodstuffs. Nevertheless, the fact that other substances may be oxidised in the muscles is shown by a series of experiments carried out by Krogh and Linhard on the performance of muscular work on low carbohydrate diets. They have shown that the respiratory quotient, or the ratio of the volume of carbon dioxide expired to the volume of oxygen retained, under these conditions is well below the respiratory quotient of unity which is observed when carbohydrate material alone is being oxidised. They conclude that fat must be oxidised in such cases where there is a deficiency of carbohydrate. It is indeed clear that here, although lactic acid must be formed in the contracting muscles, where it is really part of the mechanism, it cannot be the substance which is burned to supply the energy necessary for the recovery process. Under the conditions of carbohydrate deficiency which Krogh and Linhard investigated, and assuming that the lactic acid necessary for the contractile process can be derived only from glucose, it is obviously important—indeed essential—that this source of the acid should be conserved. Hence the tissues turn to some other substance for the energy required to keep the contractile mechanism in working order.

It is from observations of the type of those which we have just discussed that the biochemist bases the conclusion that in the muscle the predominating reaction, probably the only reaction, taking place in the presence of oxygen in which lactic acid is concerned is

Lactic acid ⟶ Glucose ⟶ Glycogen.

Nevertheless, in excised muscle and in the liver the reaction proceeds in both directions,

$$\text{Lactic acid} \rightleftharpoons \text{Glucose} \rightleftharpoons \text{Glycogen},$$

according to circumstances. Thus perfusion of a liver rich in glycogen, or in the event of the absence of glycogen from the liver, the presence in the perfusion fluid of glucose or fructose, alanine, glycerol or other substances, results in the formation of lactic acid. The reverse conversion of lactic acid to glucose has been demonstrated in the livers of depancreatised, and therefore diabetic, dogs. In the presence of alkali *in vitro*, we have seen that glucose gives rise amongst other products to lactic acid; but if air or oxygen be admitted no lactic acid formation is observed, oxidation being achieved along some other route and apparently occurring before the lactic acid has an opportunity of forming. Thus under anaerobic conditions lactic acid is formed in an alkaline solution of glucose just as it accumulates in muscle under the same conditions. Under aerobic conditions, in the former instance, lactic acid does not appear, oxidation taking some other route; in the latter case it is transformed back into sugar, whilst that portion of the sugar which is oxidised directly is broken down by an essentially different method. We shall see later (Chapter VII.) that the fermentation of sugar by yeast, which process is frequently called upon to supply analogies to the metabolic processes of complex tissues, shows many striking similarities to this dual series of reactions.

The resemblances just discussed between the two processes of glucose degradation *in vivo* and *in vitro* suggest that other possible intermediates may be common to both. Thus methyl glyoxal, $CH_3 . CO . CHO$, has been detected in the products of the action of alkali on glucose *in vitro*, and this substance and the closely similar dihydroxyacetone, $CH_2OH.CO.CH_2OH$, and glyceric aldehyde, $CH_2OH . CHOH . CHO$, when subjected directly to the action of alkali, give very much the

same results as glucose in regard to lactic acid formation. These and similar indications lead to the view that methyl glyoxal, dihydroxyacetone, glyceric aldehyde, and pyruvic acid, $CH_3 . CO . COOH$, may be all reasonably considered as intermediate products on the path of glucose oxidation. A great deal of experimental evidence has been published in which the capacity of these substances to fit into the scheme of glucose metabolism has been investigated by testing, in perfusion and other experiments, their power to give rise to lactic acid or to sugar. It will be noted that the production of lactic acid has been used as a criterion, and we have seen that there is now reason to doubt the further direct oxidation of this acid in the muscle. Nevertheless we must regard the formation or otherwise of lactic acid in a perfused organ or excised tissue generally as a legitimate indication of the capacity of the substance being tested to play a part in glucose metabolism, but it is possible that in many cases the acid may arise not directly from the substance used in the experiment, but from the glucose into which this latter may be transformed. It is exceedingly difficult to make a choice favouring any one of the substances which we have mentioned in preference to the others, but an attempt is made in what follows to summarise the type of evidence which is available.

Glyceric Aldehyde and Related Compounds.—Glyceric aldehyde is readily oxidised in the body. It is destroyed by liver tissue *in vitro*, and is converted to glucose and excreted as such by dogs rendered sugar intolerant with phlorhizin. It increases the glycogen content of perfused tortoise liver, and in experiments on the perfused liver of the dog it has given rise to *d*-glucose and *d*-sorbose. It is converted into *dl*- and *l*-lactic acids in the perfused liver, and by the washed red blood cells of the fasting dog. We may conclude from these observations that sufficient biochemical evidence is forthcoming to support the view that glyceric aldehyde may participate in the intermediate stages of glucose metabolism.

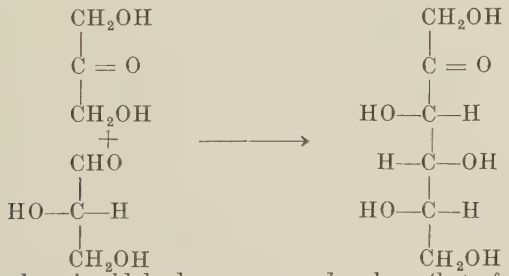
In a similar way we may consider glycerol and glyceric acid, two compounds representing simple reduction and oxidation products of the aldehyde. Glycerol fed to fasting rabbits produces a marked increase in the blood sugar level (increases up to 0·35 per cent. have been recorded), which lasts for some time. The conversion of glycerol to lactic acid and to glucose has been observed in the glycogen-free liver of the dog. Glyceric acid likewise forms glucose in phlorhizinised dog's liver.

Methyl Glyoxal (Pyruvic Aldehyde).—Methyl glyoxal gives a similar series of results. It is converted to glucose in diabetic (phlorhizinised) dogs, and to *dl*-lactic acid by perfusion through the liver, by kidney tissue, by sterile leucocytes, and by many hashed tissues. Enzymes called glyoxalases are widely distributed in animal tissues. They are capable of converting methyl glyoxal to lactic acid with great speed and in considerable quantity, if the latter is not allowed to accumulate as free acid, but is neutralised as it is formed. It is stated that an aqueous liver extract containing less than a gram of organic matter can form in this way several grams of lactic acid in the course of a few minutes. The acid produced by glyoxalases is predominantly of the *lævo*-rotatory variety.

Dihydroxyacetone.—Dihydroxyacetone is another compound which gives rise to *d*-glucose when administered to phlorhizinised dogs, and in the excised perfused liver it is converted both to glucose and to *dl*-lactic acid, whilst by the action of pig's blood corpuscles *d*-lactic acid is formed from it. Dihydroxyacetone is characterised by a remarkable ease of oxidation. Thus it reduces Fehling's solution in the cold with great rapidity, and it absorbs molecular oxygen in the presence of phosphates even more rapidly than does fructose. Furthermore, dihydroxyacetone has a well-marked anti-ketogenic action (see p. 156), and its recent production on a commercial scale has given a considerable impetus to its use in the treatment of diabetic coma where glucose is inefficient

for the purpose. Kermack, Lambie and Slater have shown that dihydroxyacetone is removed more rapidly from the blood than is glucose or fructose, and, when injected into rabbits and mice suffering from the effects of insulin hypoglycæmia (convulsions followed by coma), it is capable of effecting a recovery in the same way and at the same rate as glucose. Dihydroxyacetone is also readily utilised by diabetic individuals, and this without producing any increase in the blood sugar. Thus a diabetic subject, who was already receiving sugar to the limit of her tolerance and excreting about 1 gram of sugar per day, did not show any increased sugar elimination after the addition of 50 grams of dihydroxyacetone to the diet. That such a result cannot be explained by storage in the liver is rendered probable by the following observations. A cat was decerebrated, eviscerated, the liver was cut off from the circulation, and the renal vessels were ligated. The blood sugar level was kept constant by means of glucose transfusion at the rate of 0·15 gram per kilo per minute. On stopping the glucose transfusion and giving dihydroxyacetone in its place at the same rate, the blood sugar level began to fall, and the dihydroxyacetone disappeared rapidly and completely from the circulation. This observation also excludes the possibility that dihydroxyacetone is rapidly converted into glucose in the preparation used, and Kermack, Lambie, and Slater conclude that this substance is utilised directly. In parallel experiments sodium lactate, sodium pyruvate, methyl glyoxal, and glycerol proved to be incapable of relieving an animal from the effects of insulin hypoglycæmia. Hewitt and Reeves state that carefully purified glyceric aldehyde is also quite unable to produce any recovery in rabbits or mice suffering from hypoglycæmic convulsions or coma. The foregoing observations may have considerable significance if one admits the assumption that an intermediate product of glucose degradation *in vivo* should, under the conditions mentioned, produce the same ameliorative results as glucose itself.

The lactic acid which normally occurs in the body is the *dextro*-rotatory isomeride, and the formation of the inactive or *dl*-acid from *dl*-glyceric aldehyde, but more especially from methyl glyoxal, and dihydroxyacetone, and the production of a preponderance of the *lævo*-acid by the glyoxalases, is somewhat unexpected, and raises the question of how far one can regard these reactions which we have discussed as representing normal metabolic processes. The conversion of *l*-lactic acid into *d*-glucose, a reaction which may be carried out *in vivo*, is another case in point. It would certainly seem to involve, as Dakin has suggested, the intermediate formation of some inactive type of compound, possibly of the nature of methyl glyoxal or dihydroxyacetone. Another reaction, which has been recorded in the preceding paragraphs and which probably belongs to this category of abnormal reactions, is the formation of *d*-sorbose along with *d*-glucose from glyceric aldehyde, when the last-mentioned substance is perfused through the liver of the dog. Embden and his co-workers have suggested that this reaction may take place with the intermediate formation of dihydroxyacetone, which then reacts with the *l*-form of the glyceric aldehyde in the following manner :—

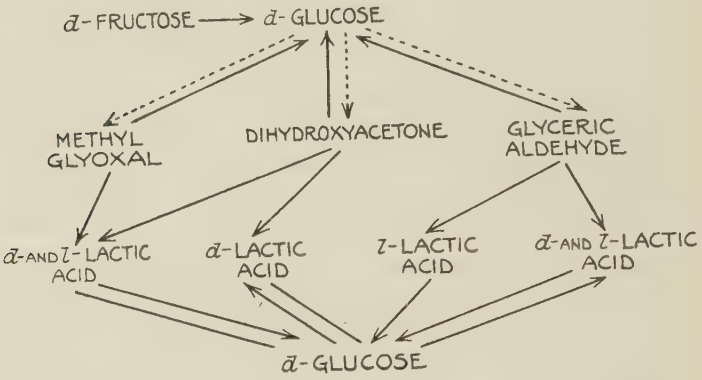


l-glyceric aldehyde *d*-sorbose (keto formula).

This suggestion is very interesting, and Embden interprets it as lending strong support to the view that dihydroxyacetone is an intermediate in carbohydrate metabolism. But we have seen that there is good reason for supposing that

sorbose is a carbohydrate which is normally not metabolised to any extent, and that it does not give rise to lactic acid in a pulp of frog's skeletal muscles. One can but record these somewhat puzzling instances of the formation of what we must regard as abnormal metabolic products without attempting an explanation.

The following scheme, slightly modified from Dakin, summarises the experimental evidence which we have just discussed, and is based on the results of transformations carried out *in vivo*, and with tissues and tissue pulps *in vitro*.



Lambie has recently published an even more comprehensive scheme. All the possibilities just outlined must clearly be considered in a full investigation of the intermediate metabolism of glucose. At present it does not seem possible to arrive at a definite decision regarding the relative parts which these compounds may play in the process.

An extension of the results of Meyerhof and his co-workers on the synthesis of carbohydrate from lactic acid in the intact frog's muscle gives a new importance to the possible *rôle* of pyruvic acid in glucose metabolism. In these further investigations Meyerhof and his collaborators have tested many other substances with the view of discovering if

alternative modes of synthesising carbohydrate in the muscle are available. As before, they recorded (1) oxygen uptake, and (2) amount of carbohydrate present, both in the control muscle and in the muscle treated with the test substance. The various substances which were tested may be classified, from the results which they gave, into three groups as follows :—

I. Without Influence on Oxygen Uptake.	II. Causing Increased Oxygen Uptake.	III. Causing Increased Oxygen Uptake and Carbohydrate Synthesis.
Glucose.	Ethylene glycol.	Pyruvic acid.
Fructose.	Glycol aldehyde.	Lactic acid.
Dihydroxyacetone.	Dihydroxymaleic acid.	
Glycero-phosphoric acid.	Glyceric aldehyde.	
Glycollic acid.	Glyceric acid.	
Alanine.	Glycerol.	
Asparagine.	Methyl glyoxal.	
	[Acetaldehyde lowered the uptake of O_2]	

Since the substances included in group I. did not increase the oxygen uptake of the muscle, no synthesis of glycogen or other carbohydrate could have occurred according to our present-day views, and this was confirmed in the determinations carried out in these experiments. The substances in group II. caused widely divergent increases in the oxygen consumption. Thus glyceric aldehyde increased it by as much as 30 per cent., whilst the other substances produced increases of an irregular and much lower order. In no case, with the exception of methyl glyoxal, did any synthesis of carbohydrate take place. Methyl glyoxal produced a slight increase in the muscle carbohydrate, a result which is ascribed to a partial conversion to lactic acid by the action of the glyoxalase of the muscle. Hence the small and variable increase in the oxygen uptake observed in most of these cases cannot be due to utilisation of the test substance in the strict sense of participation in the recovery process,

since it was not accompanied by synthetical activity on the part of the muscle. Even slight chemical stimulation of the resting intact muscle often has a marked effect in increasing its oxygen consumption, and even if the substances causing this increase were oxidised, they obviously were not utilised in the same way as is lactic acid under similar conditions. The only substance of those examined, in addition to lactic acid, which falls in group III. is pyruvic acid. This acid, like lactic acid, increased the oxygen consumption of the muscle by 50 to 150 per cent., and at the same time gave rise to a synthesis of carbohydrate. As in the instance of the lactic acid experiments, the bulk of the synthesised carbohydrate proved to be glycogen. On determining the oxidation quotient as before (the ratio of total pyruvic acid which disappears to the equivalent amount oxidised as determined from the increased oxygen consumption of the test muscle over the control), an average value of 4·7 was obtained, which is strikingly close to that given by lactic acid. We conclude from these valuable experiments that pyruvic acid, and this substance alone out of all the substances investigated, can reproduce the phenomena shown by lactic acid in the recovery process in muscle. These observations place pyruvic acid in an important position amongst the intermediate products of sugar metabolism. We have already seen that it can lay claim to a somewhat similar importance in relation to the deaminisation and synthesis of certain amino acids. The fact that the substances included in groups I. and II. in the table given on p. 115 do not participate in the recovery process in frog's muscle does not, of course, mean that they are entirely excluded from participation in glucose metabolism. Many of them may play some part in the oxidative utilisation of glucose in other ways, and in other tissues or organisms. A noteworthy instance is furnished by acetaldehyde, which, as we shall see, is an important intermediate substance in the utilisation of glucose by many micro-organisms.

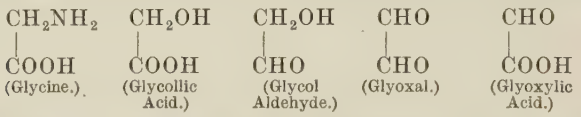
The Formation of Carbohydrate from Amino Acids

We have made reference in Chapter I. to the close relationship which exists between α-amino, α-ketonic, and α-hydroxy acids. These acids are mutually interconvertible, and since pyruvic and lactic acids both readily give rise to carbohydrate in the animal organism, it follows that α-amino acids from which these two acids may arise in the course of metabolic changes may themselves be converted into carbohydrate, and one may expect the reverse change to be accomplished with equal facility. Embden showed that the perfusion of an isolated surviving liver rich in glycogen did, in fact, yield alanine, whilst a similar perfusion of a liver free from glycogen did not produce the amino acid. The reverse aspect of this phenomenon is seen in the capacity of the diabetic or phlorhizinised animal to convert many amino acids into sugar, and we must presume that this process is one which plays an important part in normal metabolism.

Dakin has summarised these observations on the capacity of various amino acids to form glucose in the diabetic organism or in the perfused liver, and he divides the biologically important amino acids into three groups as follows : (1) those capable of furnishing glucose ; amongst these are glycine, alanine, serine, cysteine, aspartic acid, glutamic acid, hydroxyglutamic acid, proline, ornithine, and arginine ; (2) those which do not form glucose, but which give rise to acetoacetic acid ; in this group are leucine, phenylalanine, and tyrosine ; (3) a few amino acids of the " indispensable " type, such as lysine and tryptophane, which yield neither glucose nor acetoacetic acid. Dakin suggests that the reason why these two last-mentioned acids cannot be synthesised, and are therefore indispensable, lies in the fact that they do not occur in the common metabolic paths leading to and from glucose, lactic acid, and acetoacetic acid.

In general, in cases in which glucose is formed, the yield is approximately equal to that which would be expected if three carbon atoms of the glucogenetic amino acids were

transformed into sugar. Thus alanine gives a practically quantitative yield of glucose, aspartic acid gives the equivalent of three-fourths, whilst glutamic acid, proline, and ornithine yield some three-fifths of their carbon atoms as glucose. These observations again serve to emphasise the great importance which one must attach to the three-carbon compounds as intermediates in sugar metabolism. That this is not, however, the only possible means available for the synthesis of carbohydrate is shown by the fact that the two-carbon amino acid glycine yields glucose in almost quantitative amount in the diabetic organism. It is therefore somewhat perplexing to find that neither glycollic acid, glycol aldehyde, glyoxal, nor glyoxylic acid will yield glucose under conditions which lead to its formation from glycine.



Wider Aspects of the Biological Utilisation of Carbohydrates

It must not be forgotten that many of the experiments on which the modern views of carbohydrate metabolism are founded have been carried out on muscle tissue from cold-blooded animals. It is possible that in the warm-blooded mammal many of the processes which we have just considered may be modified, perhaps both quantitatively and qualitatively. But these studies, so far as they have progressed, leave us with two fundamental observations concerning what is probably the most important transformation which carbohydrate undergoes in the animal body: firstly, that hexose phosphate is the only intermediate product definitely known to be formed between glucose and lactic acid in the muscles; and secondly, that most, if not all, of the lactic acid so formed is built up again into glycogen, and is not directly oxidised. If, then, lactic acid is rejected as an intermediate product in the direct oxidation of glucose —and modern views certainly more than tend in this direction

—we must look elsewhere for the solution of our problem. It cannot be claimed that any confident answer is forthcoming. New suggestions have recently been advanced in relation to the well-known association of the last stages of fat oxidation with carbohydrate oxidation, in which it is suggested that the six-carbon glucose may be oxidised directly to a single molecule of a " ketolytic " substance without previous scission to a three-carbon compound. We shall discuss these suggestions when we deal with the oxidation of the fats.

Insulin and Carbohydrates.—This subject is dealt with in Professor Lovatt Evans' volume " Recent Advances in Physiology," and it is not proposed to elaborate the question here. It had been stated that the glucose, which rapidly disappears from the blood of normal and diabetic subjects after the injection of insulin, is not converted to fat, nor is it oxidised, nor is either hexose phosphate or glycogen formed from it. In fact, the glycogen content of the liver very frequently diminishes after administration of insulin, and during the first hour following the injection there is frequently a fall in the respiratory quotient. Experiments by Hawley and Murlin, in which continuous and prolonged records were kept of the respiratory exchange of rabbits which had received insulin, show that more than one hour after the injection of insulin there is a marked rise in the respiratory quotient. So well marked is this rise that these workers state : " Sugar burned under the influence of insulin is far greater than can be accounted for by the disappearance of sugar from the blood or from the blood, lymph, and tissue fluid at the most liberal estimate. Hence the disappearance of glycogen from the liver and other organs of normal animals, as reported by many authors." The more recent work of Best, Dale, Hoet and Marks, in which a carefully constructed " balance sheet " was prepared recording the carbohydrate exchange of an eviscerated spinal cat before and after insulin administration, proves beyond doubt that

the carbohydrate which disappears can be completely accounted for either as having undergone oxidation, or as having been deposited in the muscles as glycogen.

One may here refer to the hopes that had been entertained in relation to the discovery of insulin. It was thought that this interesting substance would serve to elucidate just precisely how sugar is utilised in the normal subject, and why its utilisation fails in the diabetic. Exceedingly interesting as the discovery of insulin is, and widespread as are the benefits accruing from its therapeutical and preventive use in diabetes, it cannot yet be claimed that it has shed any fundamentally new light on the mechanism of carbohydrate metabolism. Nevertheless a recent suggestion by Hynd merits some attention in virtue of a new conception of the first step in the normal oxidation of carbohydrate which he introduces. Thannhauser and Jenke have shown that glucosone $CH_2OH.(CHOH)_3.CO.CHO$, an oxidation product of glucose, mannose, and fructose, is utilised by diabetics, but Hynd has demonstrated that unlike dihydroxyacetone, which also is utilised by diabetics (*see* p. 112), it does not produce any alleviation of the symptoms of insulin hypoglycæmia when injected into mice. On the contrary a marked intensification of the symptoms is observed. Lactosone and maltosone, the corresponding keto-aldehydes prepared from lactose and maltose, are quite negative in their action. The glucosone effect, like that produced by insulin injection, is modified by adrenaline and pituitrin. Hynd suggests that insulin is an oxidase which catalyses the conversion of glucose into glucosone, that this is a necessary first step in the oxidation of glucose, and that therefore utilisation of glucose is lacking or diminished if insulin be absent or deficient, as it would be in the blood of pancreatic diabetics. Should the concentration of glucosone become too high, owing to excessive insulin administration, convulsions occur just as they are shown to occur when glucosone alone is injected directly. On this view the convulsions of insulin

hypoglycæmia are not to be ascribed to the lowered blood sugar *per se*, but rather to the conversion to glucosone of the sugar which disappears. Glucosone administered to normal animals produces no lowering of the blood sugar, even though the condition known as " insulin hypoglycæmia " is simulated ; on the other hand, increases of from 0·16 per cent. to 0·24 per cent. have been observed in mice. Further reference will be made to this interesting suggestion of Hynd in relation to the question of the oxidation of fatty acids (p. 157).

Metabolic Transformations of Carbohydrate in Simple Organisms

In a wider survey of the metabolic transformations of glucose the bacteria and related simple organisms, such as yeast, provide admirable laboratories for our purpose. Many simple organisms are capable of oxidising glucose completely with the production of carbon dioxide and water, but it is certain that such processes are just as complex as the similar oxidation which occurs in the higher animals, and the simple end result probably conceals a succession of complex reactions. In some cases bacterial oxidation of glucose is arrested at definite stages, and it is possible to identify intermediate products, such as gluconic and glycuronic acids. It is doubtful if either of these substances plays any appreciable part in the normal utilisation of glucose in the higher animal, since both these substances are excreted unchanged when they are administered to dogs and rabbits. It is true that glycuronic acid is frequently encountered as an excretory product of animal metabolism, but its production in such cases depends on the presence of other compounds, mostly of a toxic nature, and is to be regarded as a defensive mechanism rather than a normal feature of metabolism.

The ability to split the glucose molecule into two fragments and to produce lactic acid, by a slight atomic rearrangement, is encountered in nature in all types of organisms, from

the lowest to the highest. In general the production of lactic acid by bacteria, as for instance *Bacillus lactis aerogenes* and *Bacillus coli*, is accompanied by the formation of degradation products such as alcohol, and compounds containing four carbon atoms such as butylene glycol. We have then, as a widely distributed result of the action of living cells, the scission of the glucose molecule into two three-carbon portions, and the subsequent transformation of these fragments, by degradation and by synthesis, into both simpler and more complex substances. These reactions are therefore of great interest in biochemistry. They may be classified into two groups : (1) those which involve a scission of the glucose molecule and subsequent loss of one carbon atom ; (2) those in which scission is followed by degradation and subsequent synthesis. In the first group is included the familiar process of yeast fermentation of glucose, with the production of ethyl alcohol and carbon dioxide. In the process of alcoholic fermentation, by-products such as glycerol, succinic acid, and amyl alcohol are formed in small amount and represent some reaction other than the main one. We shall return to the question of origin of the glycerol, but meanwhile it should be noted that the amyl alcohol and succinic acid arise not from carbohydrate, but from amino acids liberated by the autolysis of yeast proteins. It is now known that, as in the case of the formation of lactic acid in the muscle, hexose phosphoric acid plays a fundamental part in the process of alcoholic fermentation, a part which we shall discuss more fully in Chapter VII. Arguing from these premises, and from the fact that traces of lactic acid can be detected in alcoholic fermentations, a theory has been suggested in which the mechanism of this process has been formulated as follows :—

$$C_6H_{12}O_6 \longrightarrow \underset{\text{(Lactic Acid.)}}{2\,CH_3 . CHOH . COOH} \longrightarrow \underset{\text{(Ethyl Alcohol.)}}{2\,CH_3 . CH_2OH} + 2\,CO_2.$$

According to this view yeast, by a simple process of decarboxylation, would convert lactic acid into alcohol.

So far no convincing support has been found for this theory, and yeast does not convert added lactic acid to alcohol by direct decarboxylation. If the formation of ethyl alcohol from lactic acid by the action of yeast does occur at all, it is by much more complex paths. Similar objections apply to theories involving the formation of glyceric aldehyde, methyl glyoxal, and dihydroxyacetone. Occasional successful fermentations have been recorded with glyceraldehyde and dihydroxyacetone, but Neuberg and Gottschalk have recently reported that, with the exception of one strain, no yeast examined by them ferments dihydroxyacetone at a rate remotely approaching that of glucose.

A theory of yeast fermentation which is more in accord with experimental facts is that which postulates pyruvic acid as an intermediate product. Neuberg and his co-workers have demonstrated the presence of a carboxylase in yeast which rapidly converts pyruvic acid to acetaldehyde, and pyruvic acid can be isolated from yeast fermentations conducted in the presence of calcium carbonate. Acetaldehyde formation can likewise be demonstrated in yeast kept under anaerobic conditions, which exclude its possible formation from alcohol by oxidation. Moreover, a mechanism exists in yeast whereby acetaldehyde and many other aldehydes are reduced to the corresponding alcohol, and it has been shown that the production of alcohol from glucose in a sterile pulp of peas also proceeds with the intermediate formation of acetaldehyde. This view of the mechanism of alcoholic fermentation involves a coupled oxidation and reduction process, which may be formulated as follows :—

$$2\,H_2O \begin{cases} \nearrow O_2 + C_6H_{12}O_6 \longrightarrow 2\,CH_3\,.\,CO\,.\,COOH + 2\,H_2O \longrightarrow CH_3\,.\,CHO + 2\,CO_2 + 2\,H_2O \\ \searrow 2\,H_2 + 2\,CH_3\,.\,CHO \longrightarrow 2\,CH_3\,.\,CH_2OH. \end{cases}$$

Neuberg and his school have recently given considerable attention to the development of these ideas, and have shown how they can be correlated with the formation of glycerol by yeast. If the reduction of the acetaldehyde is blocked,

as it is through the formation of additive bisulphite compounds when sodium sulphite is added to the fermentation mixture, glycerol is found to accumulate in considerable quantities. A similar result is obtained by the addition of "dimedon" (dimethyl *cyclo*-hexane-dione). The accumulation of glycerol is ascribed to the reduction of something else in place of the acetaldehyde in order that pyruvic acid and carbon dioxide may continue to be formed according to the coupled reaction pictured above. This process is called the "Second Form of Alcoholic Fermentation," and proceeds quantitatively according to the equation :—

$$C_6H_{12}O_6 \longrightarrow CO_2 + CH_3 . CHO + CH_2OH . CHOH . CH_2OH.$$

It was used in Germany for the large-scale production of glycerol during the war of 1914–18.

The presence of alkalis also modifies the course of alcoholic fermentation. Again glycerol accumulates and the yield of alcohol diminishes. In the presence of alkaline salts, such as carbonates, phosphates, pyrophosphates, etc., the acetaldehyde is converted by a Cannizzaro reaction into alcohol and acetic acid and, as before, the withdrawal of the aldehyde from the reaction is accompanied by the production of the molecular equivalent of glycerol. This represents Neuberg's "Third Form of Alcoholic Fermentation," and is formulated as follows :—

$$H_2O + 2\,C_6H_{12}O_6 \longrightarrow 2\,CO_2 + CH_3 . CH_2OH + CH_3 . COOH + 2\,CH_2OH . CHOH . CH_2OH.$$

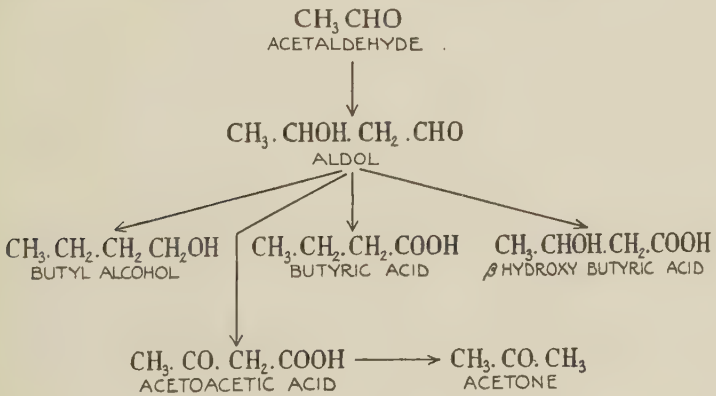
We may conclude from this work of Neuberg and his associates that there is very good reason for supposing that alcoholic fermentation involves the following steps :—

Glucose → Pyruvic acid → Acetaldehyde → Ethyl alcohol.

It is probable that the traces of lactic acid detectable in yeast fermentations are formed by reduction of pyruvic acid, a mechanism which is probably that of lactic acid fermentation itself.

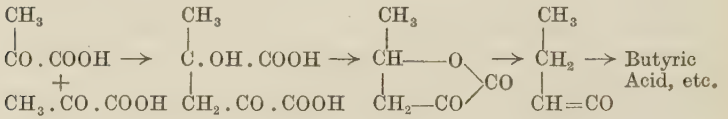
Our second group of degradation reactions in which glucose

figures is that in which the sugar is split and the resulting fragments are degraded and then converted into more complex products. This group of fermentation reactions results in the production of compounds containing four carbon atoms such as butyl alcohol, butyric acid, β-hydroxybutyric acid, butylene glycol, acetyl methyl carbinol, and diacetyl. At the same time numerous simpler compounds may be formed, for instance, acetone, lactic and acetic acids, ethyl alcohol, carbon dioxide, methane, and hydrogen. Fermentations of this type, which involve the formation of butyl alcohol, butyric acid, β-hydroxybutyric acid, and acetone—and these compounds are frequently produced together in the same fermentation mixture—are ascribed to the intermediate formation of acetaldehyde, and may therefore be regarded as modifications of those which we have already considered. In the present instances aldol is formed by the condensation of 2 molecules of the aldehyde, and this four-carbon compound is then transformed by oxidative and reductive changes into the end-products mentioned above. The series of reactions may be written as follows :—



In the scheme proposed by Smedley and Lubrzinska pyruvic acid is regarded as being the immediate precursor of acetalde-

hyde, and one molecule of each of these compounds is supposed to condense with the other to yield the unsaturated ketonic acid $CH_3 . CH = CH . CO . COOH$, from which α-crotonic aldehyde is formed. This compound is then oxidised to the corresponding acid, and from this α-β unsaturated compound the end-products are reached. Although Neuberg has been able to demonstrate the formation of acetaldehyde and of aldol by the action of yeast on potassium pyruvate, he was not able to cause any of these compounds to undergo butylic or butyric fermentation. On the other hand, he was able to ferment the lactone formed from 2 molecules of pyruvic acid, to yield butyric acid, and on this result Neuberg and Arinstein suggest the following mechanism for the process :—

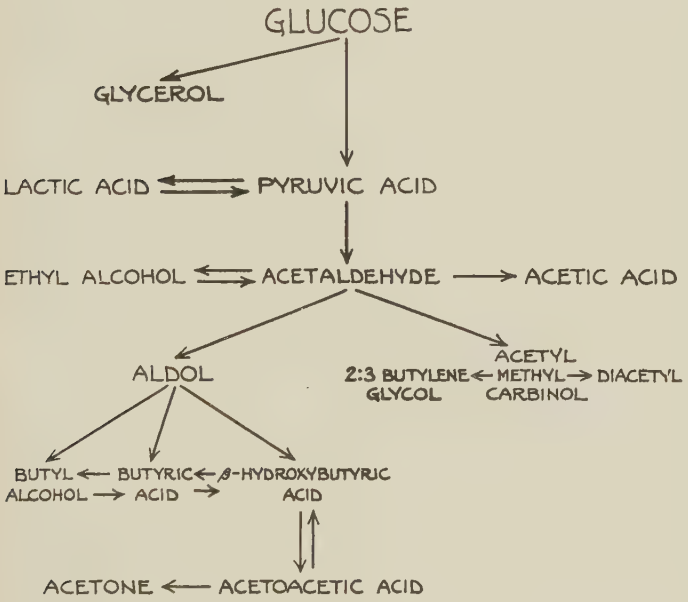


The fermentative production of the three compounds, 2 : 3 butylene glycol, $CH_3 . CHOH . CHOH . CH_3$, acetyl methyl carbinol, $CH_3 . CO . CHOH . CH_3$, and diacetyl, $CH_3 . CO . CO . CH_3$, constitutes another series of closely related reactions which occur through the activities of numerous unicellular organisms. Lactic acid is frequently encountered along with these products, and it is possible to cause acetaldehyde to accumulate in such fermentations by the addition of sodium sulphite. Lactic acid itself cannot be fermented by yeast to give any of these four-carbon compounds, whereas Neuberg and Reinfurth have shown that acetaldehyde added to yeast actively fermenting sugar is converted into optically active acetyl methyl carbinol. Part of the aldehyde which undergoes the condensation arises, however, from the sugar. Neuberg ascribes this type of synthetic reaction to the presence of an enzyme, or group of enzymes, which he calls "carboligase." As we have seen,

yeast fermentation of pyruvic acid proceeds according to the equation :—

$$CH_3 . CO . COOH \longrightarrow CH_3 . CHO + CO_2.$$

This formation of acetaldehyde takes place both in the presence of the intact yeast cells and on the addition of an extract containing the carboxylase. If a sulphite is present



in the fermentation mixture, the reaction proceeds almost wholly according to the above equation ; otherwise acetyl methyl carbinol is formed along with the acetaldehyde and carbon dioxide, thus :—

$$2\,CH_3 . CO . COOH \longrightarrow 2\,CO_2 + CH_3 . CO . CHOH . CH_3$$

This latter type of condensation reaction resembles the aldol condensation, and, indeed, acetyl methyl carbinol is an isomer of aldol. From the carbinol 2 : 3 butylene glycol would be

formed on reduction, whilst diacetyl would result from oxidation.

The fermentation reactions which we have discussed may be brought together in one comprehensive scheme given by Lemoigne, as shown on p. 127.

In general, it may be said that mixed fermentations are the rule ; thus the *B. coli* group give lactic acid and alcohol, the *B. lactis aerogenes* group lactic acid, alcohol, and butylene glycol, the *B. subtilis* group butylene glycol, acetyl methyl carbinol, and traces of acetone, whilst even such typical processes as acetic acid and yeast fermentations may give rise to the formation of acetyl methyl carbinol.

The general inference which one may draw from this survey of glucose utilisation in the lower forms of life is that acetaldehyde would seem to occupy a position of considerable importance as an intermediate product figuring in a large and diversified series of reactions. Neuberg and his school, using the sulphite and dimedon " fixation " methods, have recently endeavoured to demonstrate the formation of acetaldehyde in the tissues of higher animals, and have met with considerable success. Thus Neuberg and Gottschalk have shown that the surviving cells of a pulp of guinea-pig's liver contain acetaldehyde which can be " fixed " by the addition of calcium hydrogen sulphite. A preparation of rabbit's liver, after drying with acetone and ether, will also form acetaldehyde from pyruvic acid. A reaction which acetaldehyde, in common with many other aldehydes, undergoes in pulped tissues is a conversion to the corresponding acid and alcohol, as in the Cannizzaro reaction. Parnas has suggested the name " aldehydemutase " for the enzyme supposed to be responsible, and the frequency with which such conversions of aldehyde occur in the animal body suggests that they are of some biological importance. Other investigators have shown that acetaldehyde can be detected in blood and in surviving muscle tissue. It seems probable that in these observations the acetaldehyde has arisen from

pyruvic acid, or from some closely related compound. This, of course, does not mean that one can assert acetaldehyde to be an intermediate product of carbohydrate oxidation, since it must be remembered that pyruvic acid can be formed *in vivo* from amino acids.

Here we must leave this part of our subject at the moment, and from the mass of available evidence the reader must select for himself that theory which best accords with his own predilections.

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CHAPTER VI

THE BIOCHEMISTRY OF THE FATS

Fat is familiar to the biologist as the chief form in which excess foodstuff is stored in the tissues. In the animal its *rôle* in this respect is much more important than that of the carbohydrates. Thus in mice the reserve fat may have twenty times, and in chicks fifty times, the energy value of the reserve carbohydrate. In times of stress, such as starvation, the reserve supplies of sugar are soon exhausted, and the organism is then dependent on its reserve funds of fat for the supply of energy necessary for its maintenance. This *rôle* of the fats as the chief form of reserve fuel in the animal is sufficiently important, but at the present time the biochemist is tending more and more to ascribe to the fats a functional importance in vital phenomena of much wider significance. If we regard as fats not only those neutral compounds of glycerol and the fatty acids to which the name is familiarly assigned, but also all types of compounds in which fatty acids occur or with which fatty acids may be associated in the cell, the group assumes an importance in biology which it would not otherwise possess. Adopting this wider view, we require a term which will include all forms of "fat" of physiological significance. The International Congress of Applied Chemistry has suggested the term "lipide," and Bloor has recently recommended its adoption, giving the following classification of the compounds included in this group :—

Classification of the Lipides

Lipides are substances having the following characteristics :—

(*a*) They are insoluble in water, and are soluble in fat solvents such as ether, chloroform, and benzene.

(*b*) Their relationship to the fatty acids is that of an ester, either actual or potential.

(*c*) They are utilised by living organisms.

I. *Simple Lipides.*—Esters of the fatty acids with various alcohols.

(1) Fats—esters of the fatty acids with glycerol.

(2) Waxes—esters of the fatty acids with alcohols other than glycerol.

II. *Compound Lipides.*—Esters of the fatty acids containing groups in addition to an alcohol and fatty acid.

(1) Phospholipides or Phosphatides—substituted fats containing phosphoric acid and nitrogen ; lecithin, kephalin, and sphingomyelin.

(2) Glycolipides—compounds of the fatty acids with a carbohydrate and containing nitrogen but no phosphoric acid ; cerebrosides.

(3) Aminolipides, Sulpholipides, etc.—groups which are at present not sufficiently well characterised for classification.

III. *Derived Lipides.*—Substances derived from the above groups by hydrolysis.

(1) Fatty acids of various series.

(2) Sterols—mostly alcohols of high molecular weight, found in nature combined with the fatty acids, and which are soluble in the fat solvents ; cholesterol ($C_{27}H_{45}OH$), myricyl alcohol ($C_{30}H_{61}OH$), cetyl alcohol ($C_{16}H_{33}OH$), etc.

The most general physical property of the group of the lipides is their solubility in the so-called fat solvents. But even this property is not an absolute one. Thus lecithin will form colloidal dispersions in water which approximate to a true solution. Other members of the group are much less soluble in certain fat solvents than in others. Lecithin is insoluble in acetone, kephalin is but slightly soluble in

alcohol, and sphingomyelin and the cerebrosides are soluble in ether—a typical fat solvent—with difficulty. The melting point of the lipides is a physical property of some interest. In general it is found that the melting point of a glyceride or a mixture of glycerides is higher than that at which it solidifies on cooling. Thus beef fat has been obtained melting at 49·5° and solidifying at 36°; a butter fat may melt at 34·5° and solidify at 22·7°. It is found, however, that if the liquid fat is allowed to change to the solid form very slowly the melting and solidifying points agree to within 0·1° to 0·2°. The extent to which the liquid fat is heated above its melting point also seems to play some part in determining the temperature at which it will solidify again. The recent work of Adam, in which a study was made of the behaviour of thin films of fatty acids and their esters on the surface of water, shows that both liquid and solid films may be obtained using one and the same compound. Thus palmitic acid, normally solid up to a temperature of 62·6°, forms a liquid film on water at ordinary room temperature if the p_H of the water is 5 or less, but the film may be caused to solidify by the application of a lateral pressure of sufficient magnitude. These observations are of great interest when we call to mind the fact that many forms of fat occur in the living cells as sub-microscopic aggregates, and are present in media which are predominantly aqueous. Such phenomena, together with the delayed solidification already referred to, may help to explain why many fats in the living animal have melting points considerably above body temperature.

The Fatty Acids

The fatty acids which occur in nature may be grouped under the following series :—

(1) The saturated straight-chain series of the general formula $C_nH_{2n}O_2$, in which are included all the fatty acids containing an even number of carbon atoms from 2 to 30.

The acids with an uneven number of carbon atoms do not occur in nature. Palmitic acid with 16 carbon atoms, and stearic acid with 18, are the most important members of this series.

(2) The unsaturated series, with the general formula $C_nH_{2n-2}O_2$, *i.e.*, with one unsaturated linkage. Hypogeic acid with 16, oleic acid with 18, gadoleic acid with 20, and erucic acid with 22 carbon atoms, are members of this series. Isomers of these acids with the double bond in different positions may occur. Thus oleic acid, which is the most widely distributed fatty acid occurring in natural sources, normally has the double bond between the ninth and tenth carbon atom, that is, in the middle of the carbon chain. Two other naturally occurring isomers have been reported : one obtained from pig's liver, with the unsaturated linkage between the twelfth and thirteenth carbon atoms from the carboxyl end of the chain, and the other from rape oil. The constitution of the latter acid, called rapic acid, is unknown. Several other isomers have been prepared artificially. Another type of isomerism which this series of acids may exhibit is of the same nature as the cis-trans-isomerism of fumaric and maleic acids. The isomer of this type which corresponds to the normal form of oleic acid is elaidic acid. The conversion of oleic acid into elaidic acid is effected by treatment with the fumes of nitrous acid, or with sulphurous acid, phosphorous acid, or phosphoric acid. Brassidic acid is the cis-transisomeride of erucic acid, and is obtained in a similar way.

(3) The doubly unsaturated acids of the series $C_nH_{2n-4}O_2$. This is known as the linolic or linoleic acid series. The known acids are all isomers of an acid with 18 carbon atoms, and are found in linseed oil and in the phosphatides.

(4) The trebly unsaturated series $C_nH_{2n-6}O_2$. These acids are again found in linseed oil and the phosphatides, and likewise are isomers of a C_{18} acid. This series is known as the linolenic acid series.

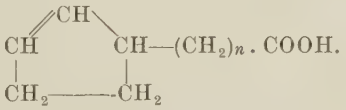
(5) The unsaturated series $C_nH_{2n-8}O_2$. The acid in this series, with 18 carbon atoms, clupadonic acid, has been isolated from fish oils, whilst that with 20 carbon atoms, arachidonic acid, has been isolated from liver and brain phosphatides.

(6) The saturated hydroxy acid series of the formula $C_nH_{2n-1}O_2(OH)$. Included in this series is the acid of the cerebroside phrenosin, namely, phrenosinic acid, to which the constitution $C_{25}H_{50}O_3$ has been assigned. If this formula is correct, it would appear that the rule which holds in the instances of the non-hydroxylated fatty acids concerning the even number of their carbon atoms does not apply in the same strict way to the hydroxy acids.

(7) The saturated dihydroxy acids of the serial formula $C_nH_{2n-2}O_2(OH)_2$. In this series we have a dihydroxystearic acid, which occurs in castor oil, and the lanoceric acid, $C_{30}H_{60}O_4$, of wool fat.

(8) The unsaturated hydroxy acids of the series $C_nH_{2n-3}O_2(OH)$. Ricinoleic acid, with 18 carbon atoms, belongs to this series. Its glyceride is the main constituent of castor oil. Isomers of this acid are known.

(9) The cyclic fatty acids. Very little is known of these acids as yet. Chaulmoogric acid and hydnocarpic acid, $C_{18}H_{32}O_2$, have the same composition as linolic acid. They occur in Chaulmoogra oil, which has recently been used as a therapeutic agent for leprosy. The constitution assigned to these cyclic acids is—



In the animal most of the more highly unsaturated fatty acids occur, not as triglycerides, but in the phosphatides, or as esters of cholesterol. Acids of a higher degree of unsaturation than that of oleic acid are not usually found in the fat

stores of the animal body, with the possible exception of the liver. In the plant many highly unsaturated fatty acids occur as triglycerides in vegetable oils, such as linseed oil. By the process of catalytic hydrogenation it is possible to convert liquid unsaturated inedible oils of plant origin, *e.g.*, cotton-seed oil, into solid fats, which are utilised in the animal body as well as the naturally hard fats. The determining factor in relation to the utilisation or otherwise of a fat is its melting point ; the higher the melting point the less likely is the fat to be metabolised.

A triglyceride may contain three different fatty acid residues, or two of these may be the same whilst the third is different, or, lastly, all three fatty acids may be the same. The naturally occurring fats seem to consist predominantly of mixed triglycerides, that is to say, the first two possibilities are more frequently encountered than is the latter. We shall see that the phosphatides, which we shall discuss in a later chapter, are constituted on similar lines, in that one of their fatty acid residues is saturated whilst the other is unsaturated. It is apparent that in mixed triglycerides of the type—

$$\begin{array}{l} CH_2OR_1 \\ | \\ CHOR_2 \\ | \\ CH_2OR_3 \end{array} \qquad \text{or} \qquad \begin{array}{l} CH_2OR_1 \\ | \\ CHOR_1 \\ | \\ CH_2OR_2 \end{array}$$

the central carbon atom is asymmetric, and therefore such glycerides might be expected to exist in optically active forms. Many attempts have been made to obtain optically active fats, but without success, unless one of the constituent fatty acids is itself optically active, as in castor oil and Chaulmoogra oil. A recent investigation of this question is that of Bergmann and Sabetay. These workers had difficulty in resolving even such markedly asymmetric compounds as glyceryl monolaurate and glyceryl

monostearate, $C_{11}H_{23}CO \cdot OCH_2 \cdot CHOH \cdot CH_2OH$ and $C_{17}H_{35}CO \cdot OCH_2 \cdot CHOH \cdot CH_2OH$ respectively. The non-success in the case of the much less markedly asymmetric triglycerides is, therefore, not surprising.

The Cerebrosides

Two representatives of these compounds, which are classed as galactolipides, are known, namely, phrenosin and kerasin. They contain the hexose sugar galactose, the base sphingosine, and a fatty acid. The fatty acid found in phrenosin is phrenosinic acid, which we have already mentioned. In kerasin its place is taken by lignoceric acid, which belongs to the normal saturated fatty acid series and has 24 carbon atoms. The two cerebrosides are of considerable interest on account of their occurrence in large amounts in brain tissue. Moreover, the combination of fatty acid and carbohydrate is not a common one in nature, nor is the hexose galactose known to occur in any other combination in the tissues. The galactose of these compounds is linked through its reducing group to the base sphingosine, and this latter is in turn linked to the fatty acid. The author and Mr. Humphreys have demonstrated that the galactose is present in its amylene oxidic form, that is, in the same form as is found in the disaccharide lactose and in stable crystalline galactose. The significance of the cerebrosides is as yet unknown.

The phosphatides are dealt with in Chapter VII.

The Metabolism of the Fats

Under certain conditions ingested fat may be hydrolysed to some extent in the stomach, as was first demonstrated by Volhard in 1900. Subsequent observation tends to show that a considerable part of the lipolytic activity of the gastric contents is due to regurgitation of pancreatic juice from the

intestine. But the whole of it cannot be ascribed to a reflux of duodenal contents. Of the residual lipase activity, some may be due to an esterase rather than to a true lipase (see p. 142), but a small amount of the latter does appear to occur in gastric juice. Thus the recent work of Willstätter and his co-workers establishes the presence in "gastric lipase" of a true lipase as distinct from an esterase. But the supposed difference in the optimal hydrogen ion concentration for the action of gastric and of pancreatic lipase is not substantiated when the preparations are carefully purified. The only difference between the two lipases which Willstätter was able to demonstrate is in their action on inactive mandelic and phenylchloracetic acid esters. Pancreatic lipase yields the *lævo*-rotatory and gastric lipase the *dextro*-rotatory acid on hydrolysis of these esters, and the two lipases would therefore seem to possess a certain degree of stereochemical specificity. Gastric lipase is destroyed in fifteen minutes by 0·02 per cent. hydrochloric acid, and it would appear that its action as a lipolytic agent cannot therefore be very important. If, however, the fat is fed in an emulsified form, as in milk, and if sufficient protein is present in the gastric contents to lower the hydrogen ion concentration considerably, fat hydrolysis in the stomach may then be fairly extensive. This is probably the case in young mammals ingesting considerable quantities of milk, and may also occur in pathological conditions in which hydrochloric acid secretion is defective.

The hydrolysis of ingested fatty material is carried out chiefly in the alkaline medium of the intestine. By the action of the pancreatic lipase in conjunction with emulsifying agents, principally bile salts and the soaps formed during hydrolysis, the neutral fats, which comprise the bulk of ingested fat, are completely hydrolysed to their component fatty acids and glycerol. These hydrolysis products are then absorbed and resynthesised to form neutral fat in the wall of the intestine. The resynthesised fat does not enter the

blood stream directly, as do other food components derived from proteins and carbohydrates, but passes into the lymphatic vessels and eventually reaches the blood stream *viâ* the thoracic duct. This method of absorption probably depends in some measure, as Bloor has suggested, on the insolubility in water of most forms of fat. There is no evidence that unhydrolysed fat is absorbed in appreciable amount. Substances of an oily nature which cannot be hydrolysed by lipase, and so rendered soluble in water for

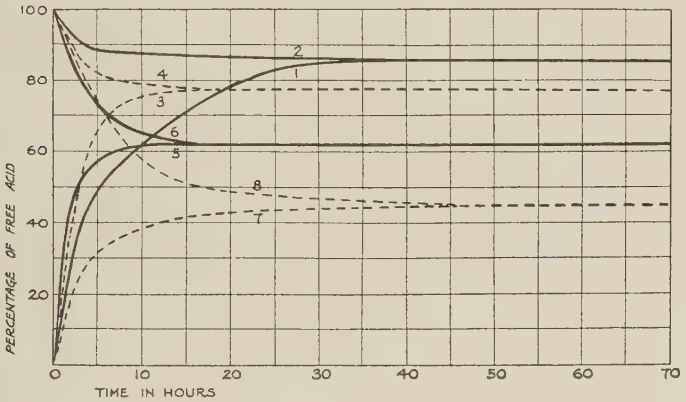


Fig. 10.—Reversibility of action of Ricinus lipase on glyceryl trioleate. (After Armstrong and Gosney.)

their passage through the epithelial cells of the intestinal wall, are not absorbed even though they are emulsified. The immediate resynthesis of the neutral fat in the wall of the intestine is brought about by the same enzyme, lipase, which produced hydrolytic cleavage of the original fat in the lumen of the intestine. The complete reversibility of the action of lipase in respect of its hydrolytic and synthetic functions has been demonstrated by Armstrong and Gosney, from whose paper the curves shown in Fig. 10 are taken. The lipase used was that of the castor bean (ricinus lipase). It was

set to act on the following systems under conditions otherwise comparable :—

(1) 1 Mol. Olive Oil Triglyceride + 9 Mols. Water.
(2) 1 Mol. Glycerol + 3 Mols. Oleic Acid + 6 Mols. Water.
(3) 1 Mol. Triglyceride + 6 Mols. Water.
(4) 1 Mol. Glycerol + 3 Mols. Oleic Acid + 3 Mols. Water.
(5) 1 Mol. Triglyceride + 3 Mols. Water.
(6) 1 Mol. Glycerol + 3 Mols. Oleic Acid.

In each of these systems the percentage of free acid was determined and plotted against time, as shown in the curves, which bear numbers corresponding to the systems given above. In systems (1), (3) and (5) the free acidity rose to an equilibrium value identical with that likewise reached in the systems (2), (4) and (6) respectively, which at the commencement of the experiment contained free glycerol and acid. These experiments demonstrate very clearly that the relative amount of water present in each system determined the final position of equilibrium reached under the action of the lipase, irrespective of whether the initial components were glycerol and oleic acid or trioleide. Curves (7) and (8) demonstrate the effect of an additional molecule of glycerol in altering the equilibrium position by favouring synthesis. The systems in these instances were :—

(7) 1 Mol. Triglyceride + 1 Mol. Glycerol + 3 Mols. Water.
(8) 2 Mols. Glycerol + 3 Mols. Oleic Acid.

Although the evidence is against the direct absorption of fat or of its hydrolysis products into the portal blood vessels, it is well known that only some 60 per cent. of the total fat which disappears from the intestine can be recovered from the thoracic duct, where it empties into the venous circulation. The fate of the remaining 40 per cent. remains somewhat of a mystery. Lee (quoted by Bloor) has recently shown that after ligation of the thoracic duct connections were established between the duct and the azygos vein or its branches, and also to the right thoracic duct.

The establishment of such additional pathways between the lymphatics of the intestine and the venous circulation may explain most, if not all, of the discrepancy already mentioned.

Fat injected subcutaneously or into the peritoneal cavity is absorbed in considerable amount. This absorption takes place through the lymphatic system and the thoracic duct.

After absorption the fat is found in the blood in the form of a fine emulsion, the particles being about 1 μ in diameter. During the post-absorptive increase in blood fat the phosphatides of the blood also show an increase, which runs roughly parallel to the increase in blood fat. Most of the increase in the phosphatides takes place within the corpuscles. It would appear that this newly-formed phosphatide is produced from the fatty acids absorbed. If this is the case, it lends strong support to the view that the phosphatides play an important part in the metabolism of the fats as a whole (see p. 150). The amount and distribution of the total lipides of dog's blood before and after feeding on a meal containing fat is shown in the following figures obtained by Bloor and Knudson (quoted by Leathes and Raper) :—

TABLE X

Amount and Distribution of Blood Lipides

	Before Feeding.	Four hours after Feeding.
In the Corpuscles.	Grams.	Grams.
Phosphatides . .	0·740	0·944
Cholesterol . . .	0·012	0·097
Glycerides, soaps, etc. .	1·598	2·669
In the Plasma.		
Phosphatides . .	0·601	0·647
Cholesterol . . .	0·216	0·243
Glycerides, soaps, etc. .	1·013	1·759

During absorption, in addition to increasing in the blood,

fat is also found to accumulate in the liver, and to a less extent in other organs, such as bone marrow, spleen, and muscles.

The passage of fat from the blood into these and other organs is not preceded by hydrolysis, such as is necessary before the fat can be absorbed from the intestine. If such a process did occur, the presence of lipases in the blood and in the tissue cells would be necessary. The available evidence is against this possibility. It is true that enzymes called lipases are widely distributed in the tissues generally, but Willstätter and his school differentiate these from true lipases. The so-called tissue lipases, or, better, "esterases," such as liver lipase, readily hydrolyse esters such as ethyl and amyl butyrate, and the glycerides of short-chain fatty acids, but do not hydrolyse the glycerides of long-chain fatty acids with anything like the same facility. Lipase, in the strict sense, seems to occur only in the gastro-intestinal mechanism.

Stored Fat and Tissue Fat

Fat occurs in all organs and tissues, but from the point of view of storage the most important "depôts" are: (1) intermuscular connective tissue; (2) the abdominal cavity; (3) subcutaneous connective tissue. The fat present in these deposits consists in great part of the neutral mixed triglycerides of palmitic, stearic, and oleic acids. It is formed more or less directly from the fat ingested in the food or from transformed carbohydrate or protein. It is possible to modify the nature of such depôt fat by altering the food-stuff. In general, the fat stored is different from that fed and is more or less characteristic of the animal in which it occurs, the differences being accountable for by alterations in the relative amounts of saturated and unsaturated fatty acids present. By feeding excessive quantities of fat it is possible to lessen the extent of the modification which the fat normally undergoes on its way from the intestine to the

stores, so that the type of fat deposited in the latter approximates to that fed. Again, when fat is being laid down on a rich carbohydrate diet, it is usually of the " hard " variety, that is, it contains a relatively high proportion of fully saturated fatty acids.

In the tissues generally there is a large amount of fat which cannot be extracted by means of the usual fat solvents (ether, chloroform, and benzene), but which may be removed by hot alcohol. Such fat cannot be seen with the microscope, nor can it be stained with the usual fat stains used by the histologist. These forms of fat consist in large part of phosphatides and cholesterol and its isomers and esters. It is possible by various means, such as the injection of diphtheria toxin, to unmask some of this hidden fat and cause it to collect in discrete and visible droplets. In the process of fatty degeneration we have a similar unmasking occurring as a pathological process, since it is well known that the degenerated organ frequently contains no more total fat than the normal organ. A characteristic difference between the fat stored in connective tissue or adipose tissue and that of tissues such as the liver, muscles, or glands is the higher degree of unsaturation of the fatty acids of the latter. More especially is this the case with liver fat, and the presence in this organ of a relatively large amount of unsaturated acids is ascribed by Leathes to the fact that the first stage of fat catabolism, namely, desaturation of the fatty acids, takes place predominantly in the liver. In starvation and in phosphorus poisoning the amount of fat in the liver undergoes an increase, and the fatty acids then found are more saturated than those normally present. There is a mobilisation of fat in the liver at the expense of the more saturated depôt fat.

It is frequently stated that the fat stores of the body are exhausted during prolonged starvation. It is true that after the exhaustion of the carbohydrate stores, which takes place during the first few days of complete starvation, the

body draws its supply of necessary energy from the oxidation of fats, and this process may continue until the stored fat is exhausted. But the recent work of Mayer and of Terroine proves that organs such as the kidney, lung, spleen, heart, etc., of animals which have been starved to death contain practically the same amount of fat as the corresponding organs of normal animals. It is the stored fat of the adipose tissue, and to a less extent that of the muscles, which is used up at need, and not the fat of the general tissue cells. The

TABLE XI

Lipocytic Coefficients of Various Animal Organs

Animal.	Lung.	Spleen.	Kidney.	Liver.	Heart.	Muscle.
Dog . .	20·0	15·5	10·5	6·8	4·6	2·2
Rabbit .	17·1	—	13·3	8·4	—	7·3
Guinea-pig	15·5	9·0	7·8	6·5	—	1·6
Pigeon .	24·1	—	9·1	7·9	3·0	1·6
Eel . .	11·2 (gills)	—	7·1	3·8	—	0·7

modern tendency is to regard the fatty material of the latter as an essential constituent of the cell structure at least as important as the proteins. It cannot be dispensed with entirely, and therefore cannot be utilised in the same way as the stored fat if the animal is to survive at all. The French school refers to the fatty acids which cannot be diminished in amount without causing death as the "constant element," whilst the fatty acids of the neutral fats which comprise the true fat reserve constitute the "variable element." One naturally associates the constant element with the cell phosphatides and with cholesterol. It is certainly rich in phosphorus, and there appears to be a definite ratio between the constant element and the cholesterol of the tissues. The ratio is expressed numerically

as $\frac{\text{cholesterol}}{\text{fatty acids}} \times 100$, and is called the lipocytic coefficient. It is characteristic for any given organ, but varies in different species. The table shown on p. 144 gives some of Mayer and Schaeffer's results for corresponding organs from various animals.

It will be observed that the lipocytic coefficients always fall in the same order in the organs of the different species investigated.

The difference between stored fat and essential fat is strikingly illustrated in the case of the brain. This organ, as is well known, does not lose weight during starvation, and as Leathes has pointed out, "that means that the organ which contains the largest amount of what may be called organised fat can dispense with none of it if it is to live."

Synthesis of Fat in Living Tissues

From the observations which we have just discussed we must regard the lipides as indispensable constituents of the living cell. The question which at once arises is the ability or otherwise of the animal organism to synthesise the necessary lipides for itself from other food constituents. This ability it appears to possess. Given an adequate supply of proteins and carbohydrate together with the necessary inorganic constituents and vitamins, young animals grow in a normal manner. It is, however, important, as we shall see in a later chapter, to ensure that the vitamins normally present in fats are supplied from some alternative source. Under such conditions fat may be entirely eliminated from the dietary, yet no harmful results ensue. One must conclude that the necessary fat is being synthesised from the other carbon-containing foodstuffs, namely, from the carbohydrate or from the protein, or from both. The evidence for the participation of these separate components of the foodstuff in the synthesis of fat will now be considered.

Synthesis of Fat from Carbohydrate.—Emil Fischer called attention to the fact that the natural fatty acids which in a quantitative sense are by far the most important are those containing 18 carbon atoms. Such acids might arise by condensation of three hexose units and subsequent oxidation and reduction. Suggestive as this analogy is, there is no experimental evidence which can be quoted in support of such a synthesis. It seems more probable from the observation that only acids with an even number of carbon atoms occur in nature that the synthesis is achieved by the successive condensation of two-carbon units. Indeed, it is stated that all the even-numbered fatty acids from butyric to stearic acids are found in milk. Direct proof of the conversion of carbohydrate into fat in living animals was first furnished in the classical experiments of Lawes and Gilbert. These need not be discussed here. Suffice it to state that subsequent observations have amply confirmed the original work. The chemical mechanism which underlies the transformation is not yet satisfactorily elucidated, but that which is most favoured by biochemists involves successive aldol condensations of acetaldehyde, with appropriate reductive and oxidative changes. The production of butyric acid by various bacteria acting on glucose and on lactic acid is well known, and the change may be formulated as follows (see also p. 127):—

$$\text{Glucose} \longrightarrow \left\{\begin{matrix}\text{Lactic acid}\\ \text{or Pyruvic acid}\end{matrix}\right\} \longrightarrow \text{Acetaldehyde} \longrightarrow$$

(aldol condensation) β-Hydroxybutyric aldehyde ⟶ Butyric acid.

In such bacterial fermentations caproic acid and small amounts of octoic and decoic acids are also found. Their presence would seem to point to further aldol condensations, starting perhaps with butyraldehyde. Such a series of reactions may well form the basis of the synthesis which we are discussing, and there are no serious experimental objections to it. There is as yet, it is true, little biological evidence

in favour of the view that lactic acid is transformed into acetaldehyde, but no such objection exists in the case of pyruvic acid, which is, as we have seen, a possible intermediary product of carbohydrate metabolism. Alternative routes for the biological synthesis of simple fatty acids, such as butyric acid, from carbohydrates have already been discussed in the previous chapter, where, too, certain aspects of the biological conversion of carbohydrate into glycerol, which is necessary for the complete synthesis of a neutral fat, have been dealt with.

Synthesis of Fat from Protein.—Many of the amino acids derived from proteins are readily transformed into carbohydrate, and since the latter may be utilised in forming fat, it is therefore natural to presume that a direct transformation of protein into fat may take place. Even under optimal conditions for this transformation, it is clear that it can never be more than partial, since only a limited number of the amino acids comprising the protein molecule can possibly give rise to sugar. Direct evidence is therefore difficult to acquire. There is no doubt that much of the earlier work, notably that of Voit, on the supposed formation of fat from protein cannot now be accepted, and in the normal course of metabolism this transformation probably does not take place to any important extent. Nevertheless the animal organism can under certain conditions synthesise fat from protein. Weinland's demonstration of this synthesis in the case of the larvæ of the fly *Calliphora* still remains unchallenged. A recent investigation by Atkinson, Rapport, and Lusk elucidates the conditions under which protein is transformed into fat in the dog. Their method of interpreting the results is best shown by a quotation from their paper :—

" If protein is converted into fat two conditions must be fulfilled : (*a*) the R.Q. must be higher than that of protein itself and (*b*) there must be a retention of protein carbon. The urinary nitrogen is usually higher in the dog when the

animal is catheterised hourly than it is when it accumulates in the bladder during a calorimeter period, and the true index of protein metabolism is accounted to be the level to which the urinary nitrogen rises when the urine is fractionated hourly on a day that the dog is given the same diet but is not in the calorimeter.

" The method of calculation of the fifth hour after giving 1,000 grams of meat to Dog XVIII. will suffice as a guide to all the experiments.

" Urinary nitrogen = 1·44 grams.

	Gram CO_2	Gram O_2.	Calories.
Equivalent of 1·44 grams of N .	13·46	12·17	38·17
Found in respiration . .	10·10	8·72	
	3·36	3·45	11·32
Calories indirect			26·85

R.Q. of deposit = 0·708.
Value of fat deposited = 11·32 calories.
Calories (indirect) = 26·85.
Calories (direct) = 27·52.
C retained = 0·92 gram = 1·2 grams of fat. = 2·3 grams of glucose (8·63 calories).
Calories if C had been retained as glycogen = 29·54.

" The respiratory quotients and the heat directly measured confirm the conception that fat and not glycogen was the form in which the carbon was deposited.

" If one makes use of this method of calculation for the average hourly heat production for the series of three experiments, one may construct Table III.

" It is evident that, computed on the oxygen absorption by the method of indirect calorimetry already outlined, the calculated heat production is almost exactly the same as it is when the computation is based upon the hypothesis that

the carbon retained is laid down in the form of fat (1 gram of C retained as fat = 12·31 calories).

"TABLE III

"*Direct and Indirect Calorimetry in Hourly Periods after the Ingestion of* 1,000 *grams of Meat*

	Experiment No.	Urine N.	Protein C to body.	Calories.		C deposited as fat.	C deposited as carbohydrate.
				Direct.	Indirect.		
Dog XVIII.	68	1·44	0·84	28·44	27·55	27·83	30·30
Dog XIX.	75	1·80	1·40	32·16	30·97	30·51	34·57
,,	77	1·80	1·02	36·46	34·83	35·25	38·29
Total .				97·06	93·35	93·59	103·16

" The agreement between direct and indirect calorimetry is not as close as one would wish. . . . The calories as calculated are 3·8 per cent. less than the calories found. However, if the carbon retention is assumed to take the form of glycogen, then the calculated heat would be 6·3 per cent. higher than the amount directly measured. The findings, therefore, favour the idea of the retention of a pabulum in the form of fat. . . . These experiments, by a new method, add another link to the chain of evidence that protein may be converted into fat. It must be remembered, however, that the conditions were exceptional, in that the animals were maintained upon a nutritive plane which would fill the glycogen reservoirs, were given meat up to the limit of their willingness to consume it, and were kept in absolute rest in a calorimeter at an environmental temperature of 25° C. Under these circumstances fragments of protein metabolism, which would ordinarily have been oxidised or converted into glucose and laid down as glycogen, found no other pathway open than conversion into fat. Under a natural diet these conditions would not exist."

We may conclude from the experiments just detailed that the ingestion of a large quantity of meat protein brings about a deposition of both glycogen and fat. Only when a very large excess of meat is fed will fat alone be retained.

The Catabolism of the Fats

When reserve fat is required for use in the tissues, it is first dealt with predominantly by the liver. The transference of fat from the adipose tissue and its mobilisation in the liver is seen in an exaggerated form in pathological conditions involving acute poisoning, and to a less marked degree in partial starvation. We have already seen that much evidence is accumulating which points to the hepatic function being exercised in two directions, namely, the conversion of the neutral fats into phosphatides and the simultaneous desaturation of the fatty acids. With regard to the first point, conversion to phosphatides, the evidence for the participation of these compounds in fat metabolism, whilst it is suggestive, is admittedly circumstantial. Incidental reference to some of this evidence has already been made in this chapter, and it will be further exemplified in Chapter VII. Here it may be summarised as follows: (1) phosphatides are universal constituents of all tissues and organs; (2) phosphatides are much more readily oxidised and hydrolysed than the neutral fats, and they mix more readily with the blood plasma and the tissue fluids; (3) the phosphatides of the blood increase in alimentary lipæmia, and a similar increase in phosphatides is observed during lipæmia in individual organs; (4) the character and amount of fat in the diet of hens influences the degree of saturation of the fats and of the phosphatides of the eggs (see Chapter VII.); (5) there is evidence that the fat of milk is derived mainly, if not entirely, from blood phosphatides; (6) the bulk of the fatty acids of the liver are present in phosphatides, and both these latter and the neutral triglycerides of the liver (which are probably present in small amounts only) are more

unsaturated than are those of the fat stores. This last piece of evidence brings us to the second point mentioned above, namely, the desaturation of the fatty acids, which takes place mainly, but not necessarily exclusively, in the liver.

The average iodine number of the fatty acids of the liver, kidney, and heart varies from 115 to 135, whilst that of the fat stores varies from 35 to 65. Direct experimental evidence of the desaturating action of the liver on ingested fatty acids has been furnished by Leathes and his associates in the following way. Cod-liver oil or herring flesh—rich sources of fatty acids with a very high iodine number—were fed to rats or cats, and it was found that the fatty acids recovered from the animal's liver had an iodine number still higher than that of the mixed acids of the oils originally fed. This result might possibly be due to a selective retention on the part of the liver of the more unsaturated acids, but further evidence advanced by Hartley shows that this explanation is most improbable. Thus the reserve fat of the pig (lard) contains both stearic acid and the normal form of oleic acid, with the unsaturated linkage between the ninth and tenth carbon atoms. In the liver of the pig is found an oleic acid with the double bond between the twelfth and thirteenth carbon atoms, and also a linolic acid with its double bonds between the ninth and tenth and the twelfth and thirteenth carbon atoms. Neither of these acids is present in lard, but both would be formed from the acids which are present, namely stearic and normal oleic, by the same change, the insertion of a double bond between the twelfth and thirteenth carbon atoms in each of these acids. A similar change occurring in the fish oils fed to animals in the experiment previously mentioned would serve to account for the increased iodine number of the fatty acids found in the liver. In addition to the foregoing evidence, the occurrence in the liver phosphatides of unsaturated acids containing up to four double bonds affords collateral support for this part of Leathes' theory.

We see, then, that there is good evidence for the view that the first stage in the catabolism of the fats is a process of desaturation, which may or may not be associated with a simultaneous conversion to phosphatides, and that the liver acts in a special way as a receiving organ for the fat which undergoes this transformation. The subsequent utilisation of the fats involves their oxidation in the tissues, and our information as to what happens between the desaturation stage and the oxidation process is not as complete as one might wish. When we pick up the thread of evidence again, as represented by the fragments of the original fat recoverable from normal metabolic processes, we find that the long chain of the fatty acid molecule has become reduced to comparatively simple dimensions. The classical work of Knoop, of Dakin, and of Embden affords a sound experimental basis for our theories as to what has been happening to the fatty acid between these two stages. The work of these investigators in relation to the process of β-oxidation, which the short-chain fragments of the original fatty acid undergo in the tissues, is too well known to require detailed repetition here. The process of β-oxidation involves the oxidative disruption of the fatty acid molecule by two carbon atoms at a time, and is therefore analogous to the mechanism by which it is synthesised. Briefly, the evidence in favour of the process of β-oxidation as representing the normal course of the catabolism of at least the shorter chain fatty acids is as follows :—

(1) Knoop found that, on administering the sodium salts of the phenyl derivatives of the lower fatty acids to dogs, he could recover from the urine either hippuric acid (benzoylglycine) or phenaceturic acid (phenylacetylglycine),

$$C_6H_5 . CO . NH . CH_2 . COOH$$

and

$$C_6H_5 . CH_2 . CO . NH . CH_2 . COOH$$

respectively. The interpretation is that derivatives of fatty acids with an odd number of carbon atoms, which yielded hippuric acid, were oxidised to benzoic acid and

subsequently condensed with glycine before excretion, whilst those derivatives of acids with an even number of carbon atoms, which were excreted as phenaceturic acid, were oxidised to phenylacetic acid, and were also condensed with glycine. The strict alternation of these two excretory products in the case of the homologous series of acids used pointed to the removal of the carbon atoms two at a time.

(2) Dakin administered phenylpropionic acid subcutaneously to dogs and cats, and found in the urine, in addition to hippuric acid, β-phenyl β-hydroxypropionic acid, benzoylacetic acid, and acetophenone, a series of substances analogous to the "acetone bodies." He also found cinnamic acid, the corresponding α-β-unsaturated acid. Dakin demonstrated about the same time that a similar oxidation of the β-carbon atom could be effected by the action of hydrogen peroxide on ammonium butyrate *in vitro* at 37°.

(3) In livers perfused with oxygenated blood containing members of the saturated fatty acid series, Embden and his co-workers found that much larger amounts of acetone were formed (from acetoacetic acid) when the fatty acid present in the blood contained an even number of carbon atoms than when one with an odd number was present.

These observations all support Knoop's original hypothesis that the fatty acids are oxidised through their β-carbon atoms. It is usually assumed that this type of oxidation proceeds with the intermediate formation of the β-ketonic acid, but we have seen that Dakin was able to detect in his experiments the formation of an α-β-unsaturated acid, and the evidence available up to the present time does not enable us to draw a distinction between these two possible types of intermediate compounds. In a recent study of the catabolism of caproic acid and its derivatives Dakin has shown that on perfusing through a surviving liver the following derivatives of the six-carbon fatty acid, namely, caproic acid itself, β-hydroxycaproic acid, β-ketocaproic acid (butyrylacetic acid), and α-β-hexenic acid (*i.e.*, the corre-

sponding unsaturated acid), the quantitative differences between the amounts of the " acetone bodies " formed in each case were insufficient to indicate which, if any, of these acids was preferentially metabolised. Dakin suggests that the acids are all readily interconvertible and are normally present in equilibrium. Nor is it possible as yet to correlate satisfactorily the process of β-oxidation with the earlier process of desaturation. It is possible that the long-chain fatty acids are broken down at their double bonds into a series of short-chain acids, and that these are then subjected to oxidation, whereby two atoms of carbon are removed at one time. Or it may be that the long chain is oxidised *in situ*, the presence of the double bonds conferring some sort of stimulus on the degradation process.

The foregoing observations are strongly in favour of the view that β-oxidation is the predominant mode of utilisation of the fatty acids in the living tissues, but the possibility that other types of oxidation may play some part in the process is by no means excluded. It will be seen that our evidence for the β-oxidation process is based largely on the behaviour of short-chain fatty acids and their derivatives in the animal or in surviving tissues. A considerable part of the evidence is founded on the formation of the " acetone bodies." We know nothing of the intermediate stages which must occur between the desaturation of the long-chain fatty acids and the production of acetoacetic acid in the course of normal metabolic processes. Dakin's observations on the action of hydrogen peroxide on the fatty acids lends welcome support for the whole theory in showing that β-oxidation may occur as a perfectly normal chemical process *in vitro*. It is all the more significant when we bear in mind the close analogy which has generally been observed between oxidations carried out with this reagent and biological processes. It is precisely for this reason that recent observations by Clutterbuck and Raper on the oxidation of the normal saturated fatty acids by means of hydrogen peroxide are of considerable interest.

These investigators have shown that the ammonium salts of hexoic, heptoic, octoic, myristic (C_{14}), palmitic, and stearic acids yield approximately equal amounts of β-, γ-, and δ-oxidation products. Special significance attaches to the fact that they find succinic acid (C_4) amongst the further oxidation products of the γ- and δ-keto-acids so formed. The process can be represented somewhat as follows :—

$$\begin{matrix} \overset{\delta}{\mathrm{R}}\mathrm{.CH_2} & \overset{\gamma}{\mathrm{CO}}\mathrm{.CH_2.CH_2.COOH} \\ \mathrm{R.CO.CH_2.CH_3.CH_2.COOH} & \longrightarrow \end{matrix} \begin{matrix} \mathrm{R.COOH} + \\ \mathrm{HOOC.CH_2.CH_2.COOH} \\ \mathrm{R.COOH} + \\ \mathrm{HOOC.CH_2.CH_2.COOH.} \end{matrix}$$

The molecule undergoes disruption between the γ- and the δ-carbon atoms if either of these atoms should become oxidised. If such a process occurred in the body the ultimate results of such a scission would be in agreement with the observations of Knoop and of Embden, for the fatty acid, in place of being degraded two atoms at a time, would lose four atoms, and the residual chain would still contain an even number of carbon atoms. Incidentally, as Clutterbuck and Raper point out, succinic acid is readily transformed into carbohydrate (*viâ* malic acid), and its production in such a way as we have outlined would help to explain the biochemical conversion of fat into carbohydrate. Clutterbuck has more recently shown that, weight for weight, minced rabbit liver is eight times as active as minced rabbit muscle in bringing about the conversion of succinic to fumaric acid, and of fumaric to malic acid. Succinic acid is also closely related to aspartic acid, the latter being the α-amino derivative of the former, and it might therefore serve to link, to a certain extent, protein catabolism with the mechanisms which we have just discussed.

Whatever the process may be by which the tissues bridge the intermediate stages between the long- and the short-chain fatty acids, we may presume that oxidation proceeds until the simple compounds which we encounter from time to time in the course of normal metabolism are reached. These are

the acids containing four carbon atoms, acetoacetic and β-hydroxybutyric acid. It may be stated in passing that it is much more probable that the latter acid arises from the former than that the hydroxy acid is an intermediate in the formation of the keto acid. At this stage a new type of oxidation is necessary to ensure the disruption of the four-carbon acid, and for its completion the presence of carbohydrate is necessary. It is for this reason that fatty acid oxidation is arrested in the advanced diabetic at the acetoacetic acid stage and this acid, together with the other " acetone bodies," is excreted in the urine. In the diabetic condition, as in starvation and after administration of phlorhizin, there is in effect a state of carbohydrate starvation, and in the instance of simple starvation the accompanying excretion of " acetone bodies " is promptly arrested by the administration of glucose. Dakin has shown that the oxidation of acetoacetic acid may be accomplished *in vitro* by the action of hydrogen peroxide, acetic, glyoxylic, and formic acids and carbon-dioxide being produced. Shaffer has demonstrated that this disappearance of the keto acid is greatly accelerated by the presence of glucose. The formation of " acetone bodies " has been called *ketogenesis*, and Shaffer ascribes to compounds such as carbohydrates *antiketogenic* or *ketolytic* functions, in that they oppose the formation of " acetone bodies " or are necessary for their oxidation. Various explanations of this correlation of fat and carbohydrate oxidation have been advanced from time to time, most of which are based on the supposed formation of hypothetical intermediate products composed of fatty acid and sugar or of bodies resulting from their decomposition. There is as yet no experimental evidence which supports these suggestions. In a recent detailed investigation of this problem Shaffer suggests that there is a quantitative relationship between the amount of acetoacetic acid and of carbohydrate being oxidised, whereby two molecules of acetoacetic acid require the simultaneous combustion of one mole-

cule of glucose to ensure their complete oxidation. If the ratio of ketogenic to ketolytic molecules undergoing oxidation in the body exceeds two, " acetone bodies " will be excreted. Much experimental support for this supposition has been obtained and reasonably accurate predictions have been made of the amount of acetone excreted by starving and diabetic subjects on diets of known composition. It must, however, be remembered that a certain proportion of the ketogenic and of the ketolytic substances is derived from proteins, and the necessary calculation involved does not yet rest on an assured basis. Moreover, these computations assume that one molecule of fatty acid gives rise to but one molecule of acetoacetic acid. One cannot state definitely that such is the case. These views have led Shaffer to make an interesting suggestion regarding the oxidation of carbohydrate (see p. 119), and we may give his views in his own words : " In the human subject the maximum antiketogenic effect of glucose corresponds to approximately two molecules of acetoacetic acid for one of glucose, which is also the ratio of glucose to keto acid in the *in vitro* reaction. But *in vitro,* each molecule of glyceric aldehyde, glycol aldehyde, glyoxal or their oxidation products likewise reacts with two of acetoacetic acid. If in the body (or *in vitro*) glucose were first dissociated into two molecules of glyceric aldehyde, we should expect that under favourable conditions it would accomplish the oxidation of (2×2) or four molecules of keto acid. But this would be twice the maximum antiketogenic effect of glucose which has so far been observed. According to this line of argument it must therefore be concluded that the six-carbon glucose is probably oxidised to a single molecule of ketolytic substance without previous splitting to a triose." Reference has already been made (p. 120) to the suggestion of Hynd that glucosone is the first step in the oxidation of glucose, and glucosone is just such a ketolytic substance as Shaffer's theory requires. It is therefore of great interest to note that Hynd finds the development of glucosone

symptoms in mice to be inhibited by a previous injection, and markedly relieved by a subsequent injection of aceto-acetic acid.

Returning to our main theme to summarise what is known concerning the oxidation of the fatty acids, we may say that the stable fatty acids present in the food and in the fat stores are rendered less stable by a process of desaturation which takes place mainly in the liver. A possible source of further reactivity is their conversion into phosphatides. The long chains of the fatty acid molecules are then shortened two carbon atoms at a time until they are reduced to the dimensions of a four-carbon compound. Further oxidation is only achieved in most animals by the simultaneous oxidation of carbohydrate. It should be noted that carnivorous animals are not so dependent on the simultaneous oxidation of carbohydrate in order to ensure complete fat oxidation as are animals predominantly herbivorous in habit.

Biophysical Aspects of the Lipides and their Significance in Biology

Until comparatively recently most biochemists have been content to regard the fats and their fatty acids simply as stores of potential energy. They certainly are of very great importance in this respect, but their utility as foodstuffs by no means exhausts their biochemical importance. The clear demonstration of the necessity for lipide material in the cell so long as it continues to live certainly points to some intimate correlation with cellular processes. As we have seen, much, if not all, of this essential lipide material is present in the cell in the form of phosphatides or in association with cholesterol. For a proper appreciation of the *rôle* that phosphatides may play in living matter, it is necessary that one should understand their chemical structure. This subject will be taken up in detail in the next chapter, but for our present purpose it is sufficient to bear in mind that, in addition to possessing great structural similarity to the neutral tri-

glycerides, they also possess one free acidic group attached to the phosphoric acid residue, and also a basic nitrogen group. They are therefore amphoteric, they have an isoelectric point at which they may be expected to show a greater physical instability than at other hydrogen ion concentrations, and they can form salt-like combinations with acids at a p_H below this point, and with bases above it. The constant occurrence of small amounts of inorganic matter (potassium, calcium, magnesium) in most phosphatides suggests that such combinations do have some biological significance. In these respects, then, the phosphatides are analogous to the proteins, and lecithin shows many of the precipitation reactions typical of the latter, more especially in regard to electrolytes. Thus salts with monovalent kations do not precipitate an aqueous emulsion of lecithin, whereas, within definite ranges of concentration, salts with di- and tri-valent kations do, trivalent kations being effective in lower concentrations than the divalent ions. It has further been claimed that the precipitating action of anions on phosphatides follows the Hofmeister series originally observed in the case of proteins, but this work probably requires revision in the light of more recent ideas on the simple valency rule, and on the effect of hydrogen ion concentration in such phenomena, ideas based largely on the work of Loeb on proteins.

Many phenomena exhibited by living cells, such as specific permeability to certain molecules and ions and impermeability to others, point to the presence of some retaining membrane surrounding the surface of the cell in the form of a definite layer. And there is a reasonable presumption that this layer is composed essentially of lipide material, into the make-up of which the phosphatides probably enter. Such a layer in any case has been the basis of a great many speculations concerning cell permeability. Lipides, when present in an aqueous solution containing electrolytes, such as we have in the cell, will form a two-phase system and,

more especially in the case of the phosphatides, the ionic equilibria at the intersurfaces of such heterogeneous systems are likely to be controlled by the Donnan membrane equilibrium. The phosphatides assist us therefore in our efforts to give a physical basis to theories of cellular activity which demand differences in ionic concentrations on the opposite sides of a limiting membrane, with consequent differences in electrical potential, osmotic and diffusion effects.

But a mere crude fatty pellicle, whether admixed or not with proteins and other substances, is quite inadequate to account for the ever-varying activities of the living cell. It seems certain that the limiting membranes surrounding the cell and also within it are much more subtly constituted. Leathes in his recent suggestive Croonian Lectures has called attention to certain aspects of the behaviour of the lipides which help us to an understanding of what the physical state of these membranes may be.

We have made a reference earlier in this chapter to the work of Adam on the structure of very thin films of fatty acids and similar substances on the surface of water. The long chain of a fatty acid molecule is very like the similar chain of a paraffin hydrocarbon except that the former terminates in a carboxyl group which confers on that end of the molecule properties which are not apparent in any other part of the molecule or in the paraffin hydrocarbon. The physical effect of this constitutional difference is at once shown by the behaviour of the two types of substances when placed in contact with water. The hydrocarbon molecules tend to cling together out of contact as far as possible with the water. They do not form a film, whereas the fatty acid molecules, although they are, like the paraffins, insoluble in water, spread themselves out over the surface of the water in the form of a very thin film which, if free to expand, assumes the thickness of but one molecule of fatty acid. If a small volume of a benzene solution containing a known amount of fatty acid be dropped upon the surface of the

water, the benzene soon evaporates and leaves a known number of fatty acid molecules in the film whose area can

FIG. 11.—Successive phases in the development of myelin outgrowths from a streak of lecithin in n/100 hydrochloric acid. (Courtesy of Professor Leathes.)

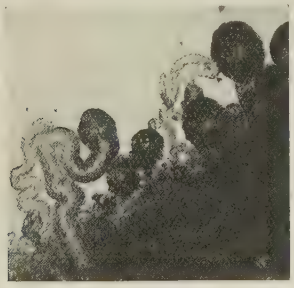


FIG. 12.—Myelin outgrowths from lecithin after 24 hours in equal parts of n/100 calcium and sodium hydroxides. (Courtesy of Professor Leathes.)

be determined by simple devices. It has been shown that the area of the film, when the molecules have been packed

close together by a lateral compressing force, corresponds to a given area per unit molecule, which area is the same for all saturated fatty acids that are capable of forming films. The vertical dimension or "depth" of the film can also be calculated, and it varies with the length of the fatty acid chain. In such fashion it can be shown that in unimolecular films the molecules are definitely oriented with

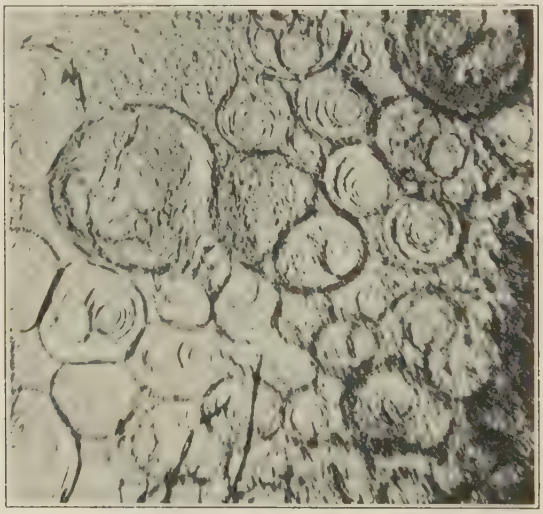


FIG. 13.—Myelin forms of lecithin containing cholesterol. (Courtesy of Professor Leathes.)

their carboxyl groups, in which the affinity for water resides, anchored, so to speak, on the surface of the water from which the rest of the molecule projects upwards. Substances present in films of this nature exhibit properties quite distinct from those which they possess in bulk. Palmitic acid remains liquid in such films even at room temperature, and at some 30° below its true melting point of 63° it passes into a physical state resembling that of a gas with its molecules all anchored at one end, as Leathes calls it, "a gas

in two dimensions instead of three." Lecithin also forms expanded "gaseous" films at temperatures as low as 5°. Cholesterol, which as a film is liquid up to 40°, causes the molecules of the expanded films of fatty acid and of lecithin to draw closer together, although they do not approximate sufficiently to form a liquid film. Liquid unimolecular films of fatty acids can be caused to solidify by the appli-

FIG. 14.—Myelin forms of lecithin containing cholesterol. (Courtesy of Professor Leathes.)

cation of a lateral compressing force. The solidification is demonstrated by small particles which were moving freely on the surface of the film suddenly becoming stationary. Even in a condensed or solid film, with the individual molecules of fatty acid packed as closely together as they are in the solid in bulk, it is of great interest to note that water molecules may readily pass through the film. An expanded or gaseous film will offer even greater facilities for such

evaporation of water through the film. In lecithin films this may be still more marked, since here the large polar group comprising the glyceryl cholyl phosphoric acid, by means of which the whole molecule is anchored, prevents close packing of the fatty acid chains.

The importance of these observations for the biochemist lies in the fact that they direct attention to what is practically a new physical state of matter at an intersurface—and living matter is one mass of intersurfaces—and particularly do they stress the fact that at these intersurfaces matter is not arranged in a random manner ; the molecules assume some definite polar configuration with regard to each other which obviously must profoundly affect their interactions. This concept of ordered array in place of random mixture may obviously be extended throughout the whole of the cell protoplasm, and simple calculations serve to show that most animal cells contain sufficient lecithin and other related substances to provide for a network of bimolecular films each with their polar groups directed towards the dispersed aqueous phase lying on each side of the film.

A further indication of this complexity as it may develop within the cell is afforded by a phenomenon closely related to those which we have just discussed, and which is seen when a streak of solid lecithin in contact with water is observed under the microscope. This consists in the development of what have been called myelin forms of lecithin—complicated outgrowths giving rise to many complex formations. Some of these are illustrated in the serial photographs taken by Professor Leathes and reproduced in Figs. 11 to 14. Leathes ascribes the formation of these outgrowths to the tendency of molecules of lecithin in contact with water to increase in number ; the behaviour of lecithin in bulk is therefore the opposite of that of oil, where the tendency is for the surface area to be kept as small as possible. The attractive force in the case of lecithin is directed from the water towards the polar glyceryl cholyl phosphoric acid

group, and this attraction exerts itself beyond the surface layer of molecules initially in contact with the water, and which are probably in an expanded form. Water molecules may therefore penetrate into the deeper layers of the lecithin with ease and there give rise to further complicated patterns. The net result is that film formation takes place in all directions, and not merely in one. The extent and form of these myelin outgrowths is modified by the presence of ions and of proteins and other substances. Some of these effects are illustrated in the photographs reproduced above.

It is clear that these phenomena of surface films and myelin formations must have some bearing on the distribution of lipides throughout living cells, and they at least serve to indicate how our older notions of the " lipoid membrane " may be expanded in a manner which is probably more nearly in accord with biological reality.

An interesting discussion of some of the physical properties of composite adsorption surfaces will be found in a recent paper by Ramsden.

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CHAPTER VII

THE BIOCHEMISTRY OF PHOSPHORUS COMPOUNDS

In the two preceding chapters reference has frequently been made to the occurrence of organically combined phosphoric acid in relation to carbohydrate and fat compounds and their metabolism. In the present chapter we shall pass on to a more detailed discussion of this type of compound and of its biological significance.

There are three chief sources of phosphorus available in normal circumstances to the animal body. These are: (1) phosphoproteins such as caseinogen of milk or vitellin of egg-yolk; (2) nucleoproteins, where the phosphorus is present in the prosthetic group in association with a carbohydrate and a purine or pyrimidine base; and (3) phosphatides, *i.e.*, lipides or fat-like bodies containing phosphorus. To a lesser extent inorganic phosphates may contribute to the bodily store of phosphorus. It is possible that this list may have to be extended in view of the discovery, reported by Kay, in cow's, goat's, and human milk of at least two, and probably three, acid-soluble organic phosphorus compounds, which are non-protein and readily diffusible. Kay gives a useful table of the forms in which phosphorus occurs in milk, thus emphasising the importance of this source, which serves as the sole foodstuff of most young growing mammals during their earliest days. These are:—

(1) Free Inorganic Phosphate.

(2) Phosphate loosely associated with caseinogen and not diffusible.

(3) Caseinogen phosphorus.

(4) Lecithin, a monaminomonophosphatide.

(5) Diaminomonophosphatide.

(6) Acid-soluble Phosphoric Ester hydrolysed by Muscle Phosphatase.

(7) Acid-soluble Phosphoric Ester hydrolysed by Bone Phosphatase.

(8) Acid-soluble organic compound unaffected by these enzymes and stable to acids.

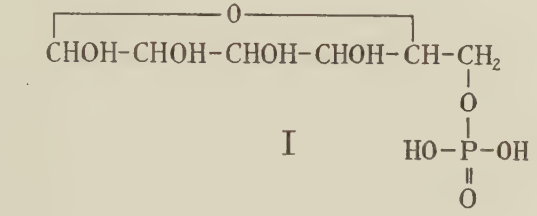
Nos. (6) and (7) are probably of the nature of hexose phosphates. It will be seen that milk contains all the forms of phosphorus of biochemical importance with the exception of nucleoprotein phosphorus. All the organic phosphorus compounds mentioned contain this element in the form of phosphoric acid linked to various organic residues.

The high content of lipide phosphorus in brain and nerve tissue and its universal distribution throughout all living cells has in the past been a marked stimulus to both investigation and speculation as to the biochemical functions of phosphorus. In recent years the interest in this element has tended to widen, and another series of phosphorus compounds, comprising the acid-soluble organic phosphorus of the tissues, has received a great deal of attention from biochemists. Included in this series are the hexose and related phosphates, and we now know that compounds of this type play a fundamental part in apparently widely different vital processes such as yeast fermentation, muscle contraction, and the ossification of cartilage. The phosphatases are enzymes which have a wide distribution in tissues and which are capable of hydrolysing and synthesising such hexose and other phosphates.

The Hexose Phosphates

The hexose phosphates are phosphoric acid esters of the hexose sugars, but we do not yet know whether they are to be regarded as derivatives of an aldose or of a ketose or of both. Assuming that both types of hexose phosphate may

exist, we illustrate below the possible structures of an aldose monophosphate (I.) and a γ-ketose diphosphate (II.), which will serve as types of these compounds :—



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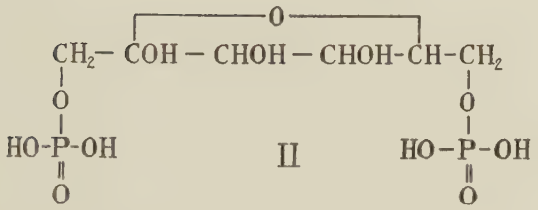


II

The phosphoric groups cannot as yet be assigned definitely to any given hydroxyl of the parent hexose, and the positions selected in the above illustrative examples are arbitrary, as are also the positions of the oxygen bridges. Three naturally occurring hexose phosphates have been definitely characterised, but it is possible, indeed probable, that others as well as pentose and triose phosphates will be found to play a part in biochemistry as important as that assigned to the known phosphates. These are : (1) hexose diphosphoric acid, $C_6H_{10}O_4(PO_4H_2)_2$, discovered by Harden and Young in the products resulting from the fermentation of sugars by yeast in the presence of inorganic phosphates ; (2) hexose monophosphoric acid, $C_6H_{11}O_5(PO_4H_2)$, obtained by Neuberg from the diphosphoric acid on partial hydrolysis ; (3) a second hexose monophosphoric acid obtained by Robison like the diphosphoric acid, by the yeast fermentation of sugars. It should be added that lactacidogen (see p. 180),

according to the most recent work of Embden and Zimmermann, has the constitution of a hexose monophosphoric acid which is said to differ from both (2) and (3). If this claim is substantiated we have here a third hexose monophosphoric acid.

Preparation of Hexose Diphosphoric and Monophosphoric Acids by Yeast Fermentation (Robison's Method).—Juice prepared from fresh pressed yeast is mixed with 10 per cent. of its weight of fructose or glucose (mannose or sucrose may also be used) and warmed to 26° in a water bath. When fermentation commences a 20 per cent. aqueous solution of $Na_2HPO_4 . 12\ H_2O$ is added from time to time in such quantity as to produce the maximum rate of evolution of carbon dioxide. When the total volume of phosphate solution added exceeds half the original volume of juice a further quantity of sugar is dissolved in the fermentation mixture. When the further addition of phosphate no longer causes any considerable rise in the rate of evolution of carbon dioxide, solid barium acetate in amount equal to the weight of the crystalline sodium phosphate added during the fermentation is dissolved in the reaction mixture, which is then rendered just alkaline to phenolphthalein with baryta, and an equal volume of alcohol is added. This precipitates the barium salts of any excess phosphate and of the two hexose phosphates together with the yeast juice proteins. After standing overnight the precipitate is filtered, washed with 70 per cent. alcohol, treated with boiling absolute alcohol, and allowed to stand in contact with the latter overnight. This renders the precipitated protein insoluble. The crude dry barium salts are ground with 10 parts of cold water, which dissolves the barium hexose monophosphate, but scarcely any of the diphosphate. The residue is ground twice with small amounts of water, filtering each time, and is then extracted with 200 parts of water, which dissolves the barium hexose diphosphate, leaving the insoluble barium phosphate and protein. The filtrate is treated with lead acetate and the

precipitated lead hexose diphosphate is separated, washed, suspended in water, and decomposed by passing hydrogen sulphide. The clear filtrate is freed from sulphide by aeration, and is finally neutralised to phenolphthalein with caustic soda. Traces of free phosphate may be removed by precipitation with magnesium acetate. The precipitation of the lead salt is repeated at least twice, and the final product is found to be free from nitrogen and to have the composition $C_6H_{10}O_4(PO_4Pb)_2$. By treating the lead salt in aqueous solution with hydrogen sulphide, filtering, and concentrating the solution *in vacuo*, the free hexose phosphoric acid may be obtained as a syrup.

The first aqueous extract of the crude barium salts is treated with basic lead acetate, and the insoluble basic lead hexose monophosphate is filtered and washed. It is decomposed with hydrogen sulphide as described above, and the acid solution is neutralised to phenolphthalein with hot saturated baryta. After filtration the precipitation with basic lead acetate is repeated. The product is contaminated with salts of succinic and other acids, by-products of the fermentation, and with a little of the diphosphate. Further purification is effected by forming the soluble barium salt, precipitating this with alcohol, and adding mercuric acetate to the filtered solution of the barium salt in 10 per cent. alcohol. From the filtrate the lead hexose monophosphate may again be precipitated. Reconversion to the barium salt and repeated precipitation of the latter dissolved in 10 per cent. alcohol by the addition of an equal volume of absolute alcohol completes the purification. After drying at 78°/2 mm. over phosphorus pentoxide it has the composition $C_6H_{11}O_5$ (PO_4Ba). The brucine salt, readily obtained from the barium salt by treatment with brucine sulphate, is easily recrystallised from methyl alcohol ($\frac{2}{3}$ vol.) and water ($\frac{1}{3}$ vol.).

Preparation of Hexose Monophosphoric Acid by Hydrolysis of the Diphosphoric Acid (Neuberg's Method).—The hydro-

lysis is best effected by means of oxalic acid, but mineral acids may be used. Calcium hexose diphosphate (13·7 grams) is dissolved in 150 c.c. of hot N-oxalic acid. After boiling for thirty minutes the solution is cooled and filtered, warmed again and shaken with an excess of barium carbonate until the reaction to litmus is no longer acid. After standing in the ice-chest the clear filtrate is concentrated *in vacuo* at 40°. After filtering a further trace of precipitate, the crude barium hexose monophosphate is precipitated by the addition of alcohol. It is purified by redissolving in water and again precipitating. When allowed to deposit slowly it is obtained in a micro-crystalline form. After drying at 105° it has the composition $C_6H_{11}O_5(PO_4Ba) + H_2O$.

Properties of the Hexose Phosphates

Hexose diphosphoric acid is feebly *dextro*-rotatory, having $[\alpha]_D + 3{\cdot}4°$. It reduces Fehling's solution after some hours in the cold, and rapidly when boiled, but its reducing power is only one-third that of an equivalent amount of glucose. When warmed with phenylhydrazine hydrochloride and sodium acetate it yields a phenylhydrazine hexose monophosphate osazone :—

$$CH:(N.NH.C_6H_5).C:(N.NH.C_6H_5).C_4H_5(OH)_3.PO_4H_2.H_2N.NH.C_6H_5,$$

which forms yellow needles melting at 151° to 152°. In the cold the product formed is diphenylhydrazine hexose diphosphate phenylhydrazone :—

$$CH:(N.NH.C_6H_5).C_5H_6(OH)_3.(PO_4H_2.H_2N.NH.C_6H_5)_2$$

The reducing power of the parent diphosphate, the presence of both phosphoric acid groups in the hydrazone, and the loss of one of these in forming the osazone, point to the lost group occupying a position adjacent to the reducing group, *i.e.*, in the 2 position if the diphosphate is a glucose derivative, in the 1 position if it is derived from fructose, which is more probable. The position of the other phosphoric acid group

remains undetermined.[1] When the diphosphoric acid is boiled with acid or with water, free phosphoric acid is produced, and from the resulting solution, which becomes *lævo*-rotatory, crystalline fructose can be isolated. The cold *lævo*-rotatory solution reduces Fehling's solution in a few minutes in the cold—a property which is also shown by the reactive, so-called "γ," sugars, but which here may be due to triose-like decomposition products. The diphosphoric acid is also hydrolysed by emulsin, and by the phosphoric esterase present in yeast juice which has been freed from co-enzyme. In both cases a *lævo*-rotatory product which is apparently fructose is formed. The hydrolytic action of acids and enzymes on the diphosphate would seem, therefore, to be the same. Morgan has recently described the preparation of α- and β-methylhexoside diphosphoric acids. Bone phosphatase (see p. 189) rapidly removes the phosphoric acid groups from the β-compound, leaving a strongly *lævo*-rotatory non-reducing substance possessing the properties of a fructoside.

Hexose monophosphoric acid (Robison's) formed along with the di-acid during yeast fermentation is also *dextro*-rotatory, having $[\alpha]_D + 25{\cdot}0°$. It forms a phenylhydrazine hexose-monophosphate osazone which melts at 139° with decomposition. A phenylhydrazone has not yet been obtained. On hydrolysing the monophosphoric acid with acids or with emulsin the rotation diminishes, but remains *dextro*. The ratio of the rotatory power of the resulting solution to its reducing power is much lower than that of solutions of pure glucose, but the ratio reducing power to phosphate liberated indicates a considerable destruction of sugar. From the hydrolysed solution glucosazone may be obtained. It is clear from the foregoing evidence that the hexose monophosphoric acid formed along with the diphosphoric acid during yeast fermentation is quite a different product and does not arise from the latter by hydrolysis, nor would it appear to be an intermediate between the sugar and the diphosphate.

[1] See Preface to the Second Edition.

The hexose monophosphoric acid (Neuberg's) obtained by hydrolysis of the diphosphoric acid is much less strongly *dextro*-rotatory than the monophosphoric acid described above, having $[\alpha]_D + 1{\cdot}5°$. It is interesting to note that, unlike the diphosphoric acid from which it is derived, and like the other mono-acid, it is fermented by living yeast and by dried yeast preparations such as zymin. It seems not improbable that this monophosphoric acid is a derivative of fructose, whereas the mono-acid formed directly during the fermentation of sugar by yeast may be a glucose monophosphoric acid. These interesting questions of structural chemistry require further elucidation.

The hexose monophosphoric acid (lactacidogen) isolated from muscle by Embden and Zimmermann has $[\alpha]_D + 29{\cdot}5°$, Its brucine salt has a lower melting point than Neuberg's brucine monophosphate, it forms an osazone which differs from that of Robison's monophosphoric acid, and its reducing power is considerably greater than that of Robison's, and much greater than that of Neuberg's compound.

The Significance of Phosphates in Yeast Fermentation.—Observations of the effect of sodium phosphate in increasing the total fermentation produced by yeast juice date from 1901, but it was not until Harden and his associates took up the investigation of this problem in 1905 that the mechanism of this effect and the fundamental *rôle* of phosphates in yeast fermentation became clear. The general effect of the addition of sodium phosphate to sugar undergoing fermentation by yeast juice is shown by the curves reproduced in Fig. 15. Curve A represents the rate of evolution of carbon dioxide from glucose undergoing fermentation by yeast juice, whilst curve B shows the effect produced on the same amount of glucose acted on by the same amount of yeast juice under parallel conditions, but with the preliminary addition of a mixture of primary and secondary sodium phosphates. The linear portion of curve A shows a carbon dioxide production of about 1·4 c.c. per five minutes. In curve B it will be seen

that the presence of phosphate raises this to some 9·5 c.c. per five minutes, but this rate falls off gradually until after an hour a uniform rate practically the same as that of curve A is attained. Curve C shows that the addition of a fresh supply of phosphate, equal in amount to the first, causes the whole phenomenon to recur. If in these curves one extrapolates the line of steady rate back to the zero

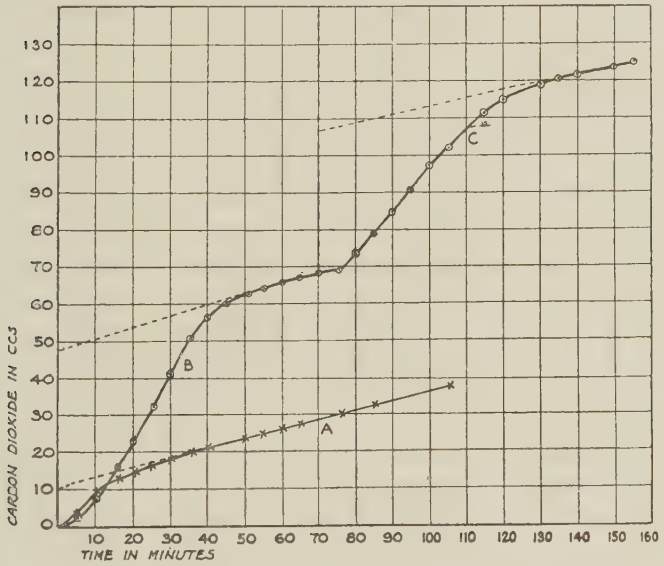


FIG. 15.—Influence of phosphate on yeast fermentation of sugar. (After Harden.)

time axis as shown by the dotted lines, it will be seen that the first addition of phosphate caused an additional carbon dioxide production of some 38 c.c. when compared with curve A, and that the second addition produced an almost identical increase in the total carbon dioxide. The calculated increase in the carbon dioxide, assuming that the effect is proportional to the phosphate added as expressed by the ratio $CO_2 : R_2HPO_4$, is, in these experiments, 38·9 c.c. By

such experiments it is possible to show that the addition of a soluble phosphate to a yeast juice-hexose fermentation mixture produces an equivalent amount of carbon dioxide and alcohol, the effect being quantitatively expressed by the equation :—

$$2\,C_6H_{12}O_6 + 2\,R_2HPO_4 \longrightarrow 2\,CO_2 + 2\,C_2H_5OH + 2\,H_2O + C_6H_{10}O_4(PO_4R_2)_2 \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (I.)$$

It will be seen that according to the above expression two molecules of hexose sugar are involved, one being converted to hexose diphosphate, whilst the other is broken down to carbon dioxide and alcohol. It is also apparent that the free phosphate is used up in forming the diphosphate, and since, when the amount of phosphate present in the materials used in a fermentation is reduced to as low a limit as possible, the rate of fermentation is proportionately diminished, it is reasonable to suppose that a supply of phosphate is indispensable for the continuation of the reaction. Whence is this to be derived ? The answer is to be found in all probability in a study of the distribution of inorganic and organic phosphate in a fermenting mixture to which phosphate has been added. Such a study reveals the following three stages in the process :—

(1) A period coincident with the increased fermentation during which the free phosphate rapidly diminishes.

(2) The uniform period following the accelerated fermentation during which there occurs no alteration in the small amount of free phosphate present.

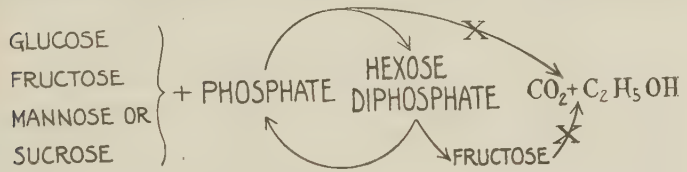
(3) The period during which fermentation lessens and finally ceases in which there is a rapid increase in free phosphate and a corresponding diminution of hexose phosphate.

An enzymic hydrolysis with production of free phosphate, similar to that occurring in stage (3), is also effected by treating hexose diphosphate with yeast juice from which the diffusible co-enzyme has been removed by dialysis, so depriving the juice of its fermenting power. The sugar

formed at the same time is found to be readily fermentable by living yeast and to possess the reactions of fructose. Assuming that this last phase mentioned above can be represented by the equation :—

$C_6H_{10}O_4(PO_4R_2)_2 + 2\,H_2O \longrightarrow C_6H_{12}O_6 + 2R_2HPO_4$ . . (II.)

Harden gives the following simple explanation of the sequence of events during fermentation. "The rapid diminution in the amount of free phosphate during stage (1) corresponds with the occurrence of reaction I. During the whole period of fermentation the enzymic hydrolysis of the hexose phosphate is proceeding according to equation (II.), Up to the end of stage (2) the phosphate thus produced enters into reaction, according to equation (I.), with the sugar which is present in excess and is thus reconverted into hexose phosphate so that as long as alcoholic fermentation is proceeding freely no accumulation of free phosphate can occur. As soon as alcoholic fermentation ceases, however, it is no longer possible for the phosphate to pass back into hexose phosphate, and hence it accumulates in the free state." The following diagrammatic scheme represents this sequence of events : —



The part played by the hexose monophosphate which is formed along with the diphosphate in this cycle is not yet clear. It may possibly represent an intermediate compound in the reaction series leading from the sugar to alcohol and carbon dioxide, in which case it might be inserted in the above scheme at the points marked X. In this connection it is suggestive that its initial rate of fermentation by yeast juice and zymin is approximately the same as that shown by a mixture of phosphate and glucose.

In a recent critical study of the equation of alcoholic

fermentation, Harden and Henley state that the ratio CO_2/total P esterified is, on the average, 0·9, indicating that, if the equation (I.) given on p. 176 be correct, about 10 per cent. of the phosphorus is esterified without evolution of CO_2. The product of this esterification is probably the monophosphate. The ratio CO_2/diphosphate formed varies considerably, but is, on the average, 2·38, that is, well above the value 2 required by equation I. This suggests that the diphosphate is originally produced in accordance with the equation, but that a part of it is subsequently partially hydrolysed with formation of the monophosphate.

Lactacidogen and the Chemistry of Muscle Contraction

Physiologists have recognised for many years that the contraction of a muscle is associated with a utilisation of carbohydrate, which in disappearing gives rise to lactic acid, and which is finally oxidised to carbon dioxide and water. The actual state of contraction of the muscle is closely bound up with the liberation of lactic acid from the muscle sugar, whilst the subsequent oxidative removal of the lactic acid occurs during the period of recovery following the active contractile state. In the absence of an adequate supply of oxygen during this second phase of recovery, the lactic acid formed during the first phase accumulates in the muscle tissue, which becomes "fatigued," or contracts with less vigour or not at all. In a long series of researches Embden, Meyerhof and their associates have established an intimate correlation between the conversion of carbohydrate into lactic acid in the contracting muscle and the presence of phosphates in the latter. Thus during contraction phosphate and lactic acid are formed in equimolar proportions. By adding excess phosphate to the muscle in an atmosphere of hydrogen all its stored carbohydrate (glycogen) may be converted to lactic acid, and as long as supplies of phosphate and glycogen or other carbohydrate are kept up, further formation of lactic acid may take place. It can also be shown

that the hexose diphosphate, which is formed during the fermentation of sugar by yeast, is converted to lactic acid and phosphoric acid by the action of the juice (muscle press juice) extracted under high pressure from fresh pulped muscle tissues. The addition of hexose diphosphate to the contracting muscle produces the same increased formation of lactic acid observed when glycogen and phosphate are added. These and similar observations led Embden and his associates to postulate the existence of an intermediate carbohydrate-phosphate compound, to which they gave the name *lactacidogen*, since in their view the formation of this compound preceded the appearance of the lactic acid in the muscle, and the acid originated from it. Indeed, they succeeded in isolating from a fresh pulp of striated muscle a substance which gave a phenylhydrazine salt of a hexosazone monophosphoric acid of the composition $C_{24}H_{31}O_7N_6P$, and having a melting-point of 148°. Later Embden and Zimmermann devised a method of extracting muscle tissue which it was thought would yield lactacidogen itself. The difficulty of isolating this substance unchanged is largely due to its contamination with other organic phosphates or with free phosphoric acid, present in the muscle or formed by the breakdown of lactacidogen during the process of isolation. It was thought to overcome this difficulty by converting the excess of phosphoric acid into lactacidogen by a process of fermentative synthesis making use of the fluoride ion, which is known to promote the formation of hexose phosphoric acid from phosphates and carbohydrates in a pulp of fresh muscle tissue or in muscle press juice. Muscle juice, immediately after extraction from the pulp by means of the high-pressure Buchner apparatus, was allowed to stand for four hours at 10° in the presence of glycogen, sodium fluoride, and sodium bicarbonate. The free phosphoric acid disappeared and, together with the added glycogen, went to form hexose phosphoric acid or, as Embden and Zimmermann supposed at that time,

lactacidogen. From the juice so treated there was isolated as the brucine salt a hexose *di*phosphoric acid identical with that formed during yeast fermentation. Naturally this observation was at first interpreted as proof of the identity of lactacidogen and yeast hexose diphosphoric acid. The latter forms a hexosazone *mono*phosphoric acid when treated in the usual way with phenylhydrazine, losing one phosphoric acid group in the process, and hence the isolation of a hexosazone monophosphoric acid in Embden and Zimmermann's earlier work seemed to fit into the general scheme. The hexose diphosphoric acid isolated from muscle, like that obtained in yeast fermentation, is converted into lactic acid and phosphoric acid by muscle press juice. In later work Embden and Zimmermann attempted to isolate lactacidogen from muscle pulp omitting the process of fermentative resynthesis, involving the addition of glycogen and sodium fluoride. To their surprise, they were unable to obtain any evidence of the presence of a hexose *di*phosphoric acid in the muscle, but obtained instead a hexose *mono*phosphoric acid only.[1] This acid is distinct from that formed along with the diphosphoric acid in yeast fermentation, and from that produced by partial hydrolysis of the latter acid (see p. 173). This new muscle hexose monophosphoric acid, which must now be regarded as lactacidogen, is converted by muscle pulp or press juice into lactic and phosphoric acids, and in the presence of sodium fluoride and press juice it yields hexose diphosphoric acid. It would therefore appear probable that lactacidogen (muscle monophosphoric acid) and Neuberg's mono acid should both be obtainable from yeast hexose diphosphoric acid on the removal of the appropriate phosphoric acid group. From the present standpoint of the Embden school

[1] The author and Mr. E. T. Waters have recently confirmed Embden and Zimmermann's statement that a hexose diphosphoric acid is obtainable from muscle pulp on the addition of glycogen and sodium fluoride, and have likewise obtained a monophosphoric acid only when these reagents were not added. (Unpublished work.)

lactacidogen is, therefore, regarded as a hexose monophosphate which is readily converted into a diphosphate in the presence of sodium fluoride and fresh muscle juice, and this diphosphate is identical with that formed in yeast fermentation. The diphosphate is not, however, formed in the muscle under normal conditions.

That there are present in the muscle enzymes which are capable of bringing about the changes discussed above has been amply demonstrated in the recent important work of Meyerhof and his school. Meyerhof has shown that it is possible to separate completely the lactic acid ferment from frog or rabbit muscle, and to obtain it in aqueous solution free from the carbohydrates of the muscle. Following a method of Buchner, it is possible to obtain, by precipitation with acetone, a dry enzyme preparation which, when redissolved, shows considerable activity. The enzyme solution shows for several hours, when glycogen has been added to it, an activity about as great as that of the minced muscle at the same temperature when calculated against muscle weight. Calculated against the dry weight of the extract, it is at least five times as great as that of the muscle pulp. A co-enzyme, which is dialysable and thermostable, can be separated from the enzyme mixture. It has been shown that the water-soluble lactic acid ferment splits hexoses only under special conditions; but, on the other hand, it readily acts upon starch, glycogen, the starch components amylose and amylopectin, and the simpler compounds derived from them, such as tri- and di-hexosans, splitting them all to lactic acid with about the same velocity. It also acts, but rather more slowly, upon the hexose diphosphoric acid of yeast, and upon the two hexose monophosphoric acids (Robison's and Neuberg's). It would appear that these reactions are not brought about by one and the same enzyme. Thus it is easy to separate from the enzyme complex the enzyme which attacks hexose diphosphoric acid. Brief heating at 38° destroys the capacity of the extract to split

glycogen and other polysaccharides, but scarcely affects its capacity to split hexose diphosphoric acid to lactic acid. Removal of the co-ferment also yields an enzyme solution which still splits hexose phosphoric acid, but which is without action on glycogen or starch. This varying behaviour towards glycogen and hexose phosphoric acid depends on the fact that removal of the co-ferment or brief heating at 38° destroys the ability of the enzyme extract to esterify the cleavage products of polysaccharides with inorganic phosphate. Thus the labile hexose formed by the cleavage of the glycogen must first be esterified with phosphoric acid before splitting and lactic acid formation can occur. As an illustration of this Meyerhof cites the following experiment. If hexose diphosphoric acid is added to the carbohydrate-free enzyme solution, lactic acid formation sets in at once, with an equivalent splitting off of inorganic phosphate (2 molecules of lactic acid for 2 molecules of phosphoric acid). If under the same conditions glycogen or starch is added, sometimes a good two minutes will elapse before lactic acid formation begins. It would now appear to be a certainty that the primary point of attack in carbohydrate cleavage in the muscle—as in yeast fermentation—is to be sought in the esterification of sugar with phosphoric acid.

So far the position is fairly clear, and we may now consider the sequence of chemical events as they occur in a contracting muscle in the light of the foregoing discoveries.

The Succession of Chemical Events in Muscle Contraction

According to present-day ideas the succession of events as they occur in a muscle contraction are as follows: The muscle glycogen combines with phosphate (presumably passing through some intermediate simpler form of carbohydrate), the resulting hexose phosphate is broken down by the muscle enzyme or enzymes with the liberation of phosphoric acid and lactic acid, and it is during this phase of acid production that the muscle fibres contract. Coincident

with the formation of lactic acid, free glucose is also formed from the glycogen. It is this latter portion of the original glycogen molecule which is directly oxidised to carbon dioxide and water, the lactic acid passing through a phase of recombination. The lactic acid formation is coincident with the mechanical change, and the modern tendency is to ascribe the actual contractile effect to a change in the surface energy of the fibres conditioned by the sudden, practically explosive, release of acid, but a difference of opinion exists as to the parts played by the two acids. Embden attaches importance to the phosphoric acid in this respect, whilst observations on the heat production of the contracting muscle point to lactic acid as the active agent. The whole contractile phase, which includes the onset and maintenance of the contraction, and the relaxation to the normal resting condition, proceeds with a relatively small heat output and can take place in the entire absence of oxygen. It is therefore called the *anaerobic* phase. During the recovery or *aerobic* phase which follows the relaxation, and which is only completed in the presence of oxygen, there is a prolonged heat production. The uptake of oxygen during this phase corresponds to the oxidative removal of the lactic acid formed during the first phase. But this acid is not directly oxidised to carbon dioxide and water ; indeed the oxygen absorbed is only one-fourth of that required to account for the oxidation of the lactic acid. It is resynthesised to hexose phosphate and ultimately, as Meyerhof and others have shown, to glycogen, whilst the phosphoric acid is liberated during the last stage of this synthetic process, and therefore becomes free to participate in the chemical events of the next contractile cycle. The production of carbon dioxide during the aerobic or recovery phase proceeds from the oxidation of the free glucose formed along with the hexose phosphate, and which represents one-fifth part (approximately) of the glycogen molecules taking part in the cycle. We have then in the contracting muscle a succession

of events of a cyclic nature, in the course of which one-fifth of the glycogen is completely oxidised to carbon dioxide and water, while the other four-fifths, passing through a reversible process whereby they are converted to hexose phosphate and lactic acid, are eventually reconstituted as glycogen and passed on to subsequent cycles. These successive and reversible reactions have been expressed by Meyerhof in a series of chemical equations which, although they cannot be accepted too literally, nevertheless serve as a useful basis for the theoretical aspects of the chemistry of muscle contraction. These equations, modified to accommodate the view that lactacidogen is a hexose monophosphate, are :—

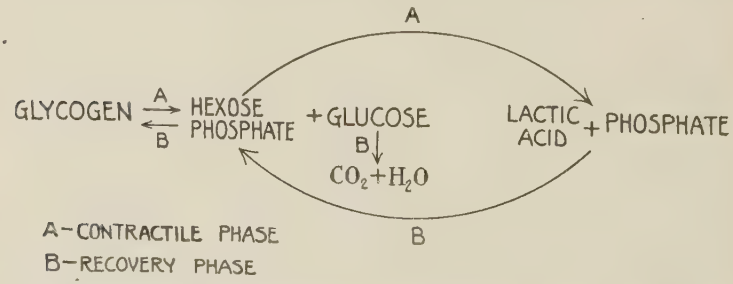
(1) *The Anœrobic or Contractile Phase.*

$$5/n(C_6H_{10}O_5)_n + 5H_2O + 4\,K_2HPO_4$$
$$= 4\,C_6H_{11}O_5(PO_4K_2) + C_6H_{12}O_6 + 4\,H_2O$$
$$= 8\,C_3H_6O_3 + 4\,K_2HPO_4 + C_6H_{12}O_6.$$

(2) *The Aerobic or Recovery Phase.*

$$8\,C_3H_6O_3 + 4\,K_2HPO_4 + C_6H_{12}O_6 + 6\,O_2$$
$$= 4\,C_6H_{11}O_5(PO_4K_2) + 6\,CO_2 + 10\,H_2O$$
$$= 4/n(C_6H_{10}O_5)_n + 4\,K_2HPO_4 + 6\,CO_2 + 10\,H_2O.$$

A schematic representation of the events in the above series of equations may be drawn up as follows :—



Such a scheme, when compared with that given on p. 177 for the processes of yeast fermentation, serves to emphasise the essential similarities of the two phenomena. We have in both a reversible cycle in which the hexose phosphate

plays an indispensable part, although in neither case is it directly transformed into the end-products. In yeast fermentation the carbon dioxide and alcohol are formed from the fermentable sugar produced along with the hexose phosphate, whilst in the contracting muscle the carbon dioxide also arises from the sugar simultaneously produced with the hexose phosphate. It is an impressive fact that Nature should have devised such fundamentally similar mechanisms for two apparently dissimilar processes, the one revealed by one of the lowest unicellular plants, the other by the highly organised muscles of the highest animals.

From the account given in the preceding pages it will be seen that around the chemical and physical events coincident with and following muscle contraction, so far as we know them, a consistent theory has been developed; but from recent discoveries it would seem that the tale is by no means complete. During the past year certain nitrogenous organic compounds of phosphoric acid have been found in muscle, and in consequence it would seem probable that some *rôle* will have to be provided for these nitrogen compounds in this field of biochemistry.

In 1847, Liebig first isolated inosinic acid from muscle. Reference to p. 55 will show that this acid is a compound of hypoxanthine, *d*-ribose, and phosphoric acid. Very recently Embden and Zimmermann have obtained from muscle pulp, in addition to lactacidogen, adenylic acid, a compound similar to inosinic acid, but having adenine, the mono-amino compound corresponding to hypoxanthine, in place of the latter. It would seem that in the muscle inosinic acid arises from adenylic acid by deaminisation, and it is possible that this deaminisation process may have some relationship, as yet unknown, to the processes of muscle contraction. Thus a release of ammonia coincident with contraction, and therefore with the production of lactic and phosphoric acids, might play an important part in the buffer mechanisms of the muscle. There is yet another new aspect of our subject

which must be considered. Eggleton and Eggleton have reported the presence in frog's muscle of a substance which they named phosphagen, which is very unstable in acid solution and which disappears completely when the muscle enters into a state of rigor. The same substance has been isolated by Fiske and Subbarow, who state that only a small portion of what has been regarded as inorganic phosphate in voluntary muscle is actually inorganic. The bulk of it consists of an unstable compound of creatine and phosphoric acid, which is hydrolysed on stimulating the muscle to contract, and which is resynthesised when the muscle is permitted to recover. This highly interesting discovery may have far-reaching consequences in muscle physiology, since it may possibly lead to the provision of a much-needed function for creatine, long recognised as a constituent of voluntary muscle, but for whose presence there no satisfactory explanation has yet been advanced.

The Chemistry of Ossification

Dry, marrowless bone consists of some 60 per cent. of calcium and magnesium phosphates and carbonates deposited on a matrix of mucoids, collagen, and a small amount of other proteins. This matrix therefore is closely similar to the cartilage from which the bone is derived by the processes of ossification. Of the inorganic constituents of bone, calcium phosphate is the most important, comprising some 85 per cent. of the total inorganic matter, and therefore investigations of ossification processes have largely centred round the problem of the deposition of this insoluble salt. In bone it is probably present as a complex phosphate of the type of *hydroxyapatite*, $(Ca_3P_2O_8)_3 . Ca(OH)_2$, but for our purposes it will be sufficient to consider the tribasic phosphate, $Ca_3(PO_4)_2$.

The problem of the deposition of calcium phosphate during ossification has always been a difficult one, and it is natural that the capacity which proteins possess of fixing both calcium and phosphate in a non-diffusible form has been

made the basis of theories of ossification. Freudenberg and György are present-day exponents of such a theory, their view being that a reversible fixation of calcium by the tissue proteins is the essential mechanism. The capacity of the proteins to fix calcium and phosphate is illustrated by the results of György, who found that of the total calcium and inorganic phosphate of cow's milk some 30 to 40 per cent. is indiffusible, and that this steadily diminishes with increasing hydrogen ion concentration until at the isoelectric point of casein all the calcium and inorganic phosphate are dialysable. The same increase in the diffusible calcium and phosphate was observed during the tryptic digestion of milk at a constant p_H. After the tissue proteins begin to fix calcium the metabolic activity of the tissue diminishes and the fixed calcium reacts with phosphate and carbonate to give complex protein-calcium-phosphate-carbonate compounds, from which the carbonate is slowly eliminated by local tissue acidity. The excess calcium phosphate is then split off, leaving the original active group of the protein free to combine with more calcium. Such theories lack the support of experimental evidence obtained at the actual seat of ossification, but they may possibly play an important part in the slow calcification of tissues, such as arteries, with advancing age.

It appears that the concentration of diffusible calcium, phosphate, and carbonate in the blood plasma, from which the bone constituents must ultimately be derived, is above that corresponding to the solubility of calcium phosphate and carbonate, and it has been suggested that these are present in the plasma in the form of a metastable supersaturated solution which might deposit the solid phase of these salts on coming in contact with them in the bone. But as Robison points out, it is not justifiable to regard plasma as such a supersaturated solution until we know more concerning the factors which control the degree of ionisation and solubility products of the various components of such complex systems. Regarding the plasma or the tissue fluids as simple saturated

solutions in which we have the system solid phosphate in equilibrium with the dissolved salt, the conditions are met by the equation :—

$$\underset{\text{(Ionised.)}}{3\,Ca^{\cdot\cdot} + 2\,PO_4'''} \rightleftharpoons \underset{\text{(In solution unionised.)}}{Ca_3(PO_4)_2} \rightleftharpoons \underset{\text{(Solid.)}}{Ca_3(PO_4)_2}.$$

An increase in the concentration of either $Ca^{\cdot\cdot}$ or PO_4''' would cause the reaction to proceed towards the right and result in the deposition of solid calcium phosphate, and conversely a decrease in the concentration of either of these ions would be counterbalanced by some of the solid phosphate passing into solution and so causing the reaction to move towards the left. According to the mass action law,

$$[Ca^{\cdot\cdot}]^3 \times [PO_4'''']^2 = K[Ca_3(PO_4)_2]$$

and since we are dealing with a salt of minimum solubility,

$$[Ca^{\cdot\cdot}]^3 \times [PO_4'''']^2 = K, \text{ or the solubility product.}$$

Hence solid calcium phosphate will be deposited when the product of the concentrations of the calcium and phosphate ions becomes greater than the value of the solubility product of calcium phosphate. There is no reason to suppose that the deposition of calcium phosphate in bones can be expressed in quite such simple terms, since, as has been mentioned, hydroxyapatite and not tribasic calcium phosphate is laid down in the bone ; but these considerations illustrate the fundamental point. A shift of equilibrium towards the right in the above equation followed by a deposition of solid calcium phosphate would result from a local increase in the phosphate ion concentration, and a mechanism whereby such an increase can be brought about locally in ossifying cartilage, bone, and teeth has been revealed in a series of investigations carried out by Robison and his associates.

The Rôle of Organic Phosphates in Ossification

In an examination of the action of the enzymes present in various tissue extracts on the soluble calcium and barium salts of hexose monophosphoric acid from yeast fermenta-

tions it was found that a precipitation of the sparingly soluble calcium or barium phosphate occurred as the substrate became hydrolysed. This observation suggested to Robison the query whether some such reaction might be concerned in the deposition of calcium phosphate during the ossification of cartilage. It was found that an enzyme, which we can call a phosphoric esterase, capable of hydrolysing the salts of hexose monophosphoric acid (obtained from yeast) was present in many tissues, but more especially does it occur in ossifying bone and teeth. The distribution of the enzyme is given by the following table, which shows the percentage of (*a*) potassium hexose monophosphate, and (*b*) potassium glyceromonophosphate, hydrolysed by a 5 per cent. aqueous extract of rat tissues in the course of eighteen hours at 37° and p_H 9–9·2, and the percentage of (*c*) potassium hexose monophosphate, and (*d*) potassium hexose diphosphate hydrolysed in another series of experiments by aqueous extracts of rabbit tissues in the course of forty hours at 37°, the p_H being uncontrolled :—

TABLE XII

Hydrolysis of Organic Phosphates by Tissue Extracts
(*After Robison.*)

Tissue.	Percentage of Phosphoric Esters Hydrolysed.			
	(*a*)	(*b*)	(*c*)	(*d*)
Bone cartilage (epiphyses) .	92	—	95	89
Rib cartilage (unossified) .	2	—	8	54
Kidney	71	83	41	79
Liver	10	8	22	66
Spleen	17	20	22	50
Pancreas	1	3	6	23
Muscle	—	9	1	50
Blood	—	—	0	0

The range of activity of the enzyme or enzymes at various hydrogen ion concentrations is shown in the following table :—

TABLE XIII

Activity of Tissue Phosphoric Esterases at Varying p_H (*After Robison.*)

Tissue.	Ratio of Amounts of Ester Hydrolysed at p_H.		
	7·3	8·3	9·3
Bone	1	3·5	5·5
Teeth	1	3·7	5·8
Kidney	1	3·0	5·1
Liver	1	0·6	1·0
Spleen	1	2·0	3·2
Pancreas	1	0·2	1·5

From these results one may conclude that there is present in these tissues an enzyme capable of hydrolysing both hexose and glyceromonophosphoric acids, that it is present notably in ossifying bone, teeth, and kidney, that it is present only in traces in unossified cartilage, and that the enzyme responsible for the hydrolysis shown by the liver, spleen, pancreas, and muscle extracts is probably different from the bone enzyme, since the latter shows a steadily increasing activity as the p_H rises from 7·3 to 9·3, whereas the enzymes present in the other four extracts do not. In these experiments the enzyme solutions were kept sterile by the addition of chloroform, and the slight amount of spontaneous hydrolysis shown by the phosphoric esters was checked by controls and has been deducted. A further conclusion which one may make from these results is that the capacity of a given tissue extract to hydrolyse the monophosphoric ester does not vary in the same way as its capacity to act on the diphosphoric ester. After Robison's discovery of this bone

enzyme Takahashi was able to show that aqueous extracts of the femur also hydrolysed Neuberg's potassium hexose monophosphate obtained by partial hydrolysis of the diphosphate from yeast, and which we have seen to differ from the monophosphate obtained directly from yeast. The bone enzyme therefore appears to act indifferently on monophosphoric esters, but it is interesting to note that it has no action on lecithin or on the blood phosphatides, although the former is a derivative of glyceromonophosphoric acid.

While the evidence given above is highly suggestive of the part played by the monophosphoric esterase in bringing about a local increase of phosphate ions and so causing a deposition of solid phosphate in actively ossifying centres, it is not conclusive until it can be shown that there is a definite correlation between the initiation of ossification processes and the appearance of the active enzyme. This has been done by investigating the distribution of the phosphoric esterase in human fœtuses and young infants, with the result that in purely cartilaginous structures the enzyme is found to be absent, and in partially ossified bones it is found only at the ossification centres. Robison therefore concludes " that the production of the enzyme is a part of those cellular activities which result in the formation of bone."

The range of optimum activity of the enzyme is an interesting one. It shows a broad optimum from p_H 8·4 to 9·4. More recently Martland and Robison have shown that the activity of the enzyme increases with the alkalinity up to p_H 9·4, but above p_H 8·4 this is offset by the gradual inactivation of the enzyme. This p_H range is usually regarded as being above the normal limits of hydrogen ion concentration of the tissues generally, but a localised increase of p_H to this range is not improbable, and in any case instances are not lacking of enzymes with optima considerably outwith the small range of the blood p_H, but which nevertheless play important parts in tissue processes. Acting on glycerophosphoric acid, Martland and Robison have shown that the

rate of action of the enzyme is greatly retarded by inorganic phosphate even in low concentration, but is not appreciably affected by similar concentrations of the other hydrolysis product—glycerol. In high concentrations of glycerol and in the presence of inorganic phosphate, synthesis of phosphoric ester takes place. Synthesis has also been observed with mannitol, glycol, glucose and fructose.

The Problem of Rickets in Relation to the Bone Enzyme

As we shall see in Chapter IX., when the growing animal is deprived of the anti-rachitic vitamin D, present in cod-liver oil and other natural fats, ossification becomes defective. Attempts have been made to correlate this metabolic disturbance with a defect in the calcium or phosphate balance. There is, however, no reason to suppose that the total calcium of the blood is appreciably altered in rachitic children or animals. Although rickets is sometimes associated with a low content of inorganic phosphate in the blood the association is not frequent enough to be regarded as specific. The problem of rickets would seem to lie deeper than a mere quantitative deficiency of the inorganic constituents of bone, a view which is supported by the histological picture of the rachitic bone. It is obviously a matter of interest to examine the enzymic activity of bone extracts obtained from animals affected with rickets. Such an examination reveals the presence of the active enzyme in amounts no less than those occurring in normal animals, and in some cases more active preparations were obtained from the rachitic animals than from the normals. Rickets therefore cannot be ascribed to a deficiency of the monophosphoric esterase. It can, however, be shown that imperfectly calcified bones from young rats show extensive deposition of calcium phosphate if they are immersed in solutions of calcium hexose or glyceromonophosphates, whereas no such deposition occurs in a solution of disodium phosphate under similar conditions. It appears therefore that the causation of rickets might be found in a

deficiency of the phosphoric esters from which the phosphate of ossifying cartilage is derived. It is obviously difficult in these experiments, necessarily carried out *in vitro* on bones out of contact with their normal environment, to be quite certain how far these methods reproduce conditions in the living animal. In any case attempts to demonstrate in rachitic animals a deficiency of the phosphoric esters of the blood which are hydrolysed by the bone enzyme have not yet led to conclusive results. Korenchevsky and Carr have, however, been able to show that sodium glycerophosphate, but more especially calcium glycerophosphate, when injected subcutaneously into rats fed on a rachitic diet, produces a marked increase in the extent of calcification of their skeletons. The superiority of the calcium over the sodium salt in this respect suggests that a deficiency of some form of calcium is at least a contributory cause of rickets. It seems highly probable that separate mechanisms exist for supplying the calcium and the phosphate required for ossification, and it may be that the pathological disturbances associated with rickets do not affect both these mechanisms in the same way or to the same extent. A further point worthy of note here is that ingested calcium glycerophosphate has no effect on the calcium retention of rachitic animals, a result which accords with Robison's demonstration of the presence in the intestinal mucosa of an active monophosphoric esterase in amount similar to that of the kidney. It would seem that both the absorption from the intestine and the excretion *viâ* the kidney of the unhydrolysed phosphoric ester are safeguarded against, so that the necessary ester or esters must be synthesised by the tissues, possibly by the liver.

The Acid-soluble Organic Phosphorus of the Blood.—The problems which we have considered raise the further question of the supply of the necessary monophosphoric esters to the ossifying tissues. This must be through the blood supply, and it is an obvious step to investigate the occurrence and distribution of such esters in the blood. The acid-soluble

organic phosphate of blood occurs almost wholly in the corpuscles. The following details are given by Bloor of the phosphorus distribution in human blood :—

TABLE XIV

Phosphoric Acid of Blood in Milligrams Per Cent.

	Total.	Lipide.	Inorganic.	Acid-soluble Organic.
Corpuscles .	248·5	56·8	17·2	160·3
Plasma . .	34·1	23·5	9·9	1·5

Included in the lipide phosphoric acid fraction are lecithin and kephalin, but although these contain a glyceromonophosphoric acid residue they are not acted on by the phosphoric esterase of bone, as has been mentioned already, and therefore these lipides presumably can play no direct part in the ossification processes which we are considering,[1] although they may form a source of phosphoric acid for the hexose phosphate and so participate indirectly, as indeed we shall see is probably the case in the embryo chick (see p. 208). The fraction of the blood phosphorus which is of importance in relation to the present discussion is the acid-soluble organic phosphorus. The curves reproduced in Fig. 16 represent the results obtained by Kay and Robison on hydrolysing the acid-soluble organic phosphorus compounds present in deproteinised filtrates from rabbit's blood. Curve A shows the rate and extent of hydrolysis obtained with the bone enzyme at 37·5° and at p_H 9·3, whilst curve C shows the hydrolytic action of n/5 sulphuric acid at 100°. A curve (B) showing the hydrolytic action of the phosphoric esterase of bone on sodium hexosemonophosphate is also included in the figure. It is clear that whilst the sulphuric acid eventually

[1] The glycerophosphatase present in takadiastase appears, however, to act on lecithin. (Akamatsu, *Biochem. Z.*, 1923, **142**, 186.)

hydrolyses all the acid-soluble phosphoric esters of blood, the action of the enzyme ceases when some 25 per cent. of the esters have been hydrolysed. In other experiments the results vary between 14 and 36 per cent. of the total acid-soluble organic phosphorus. One might therefore conclude

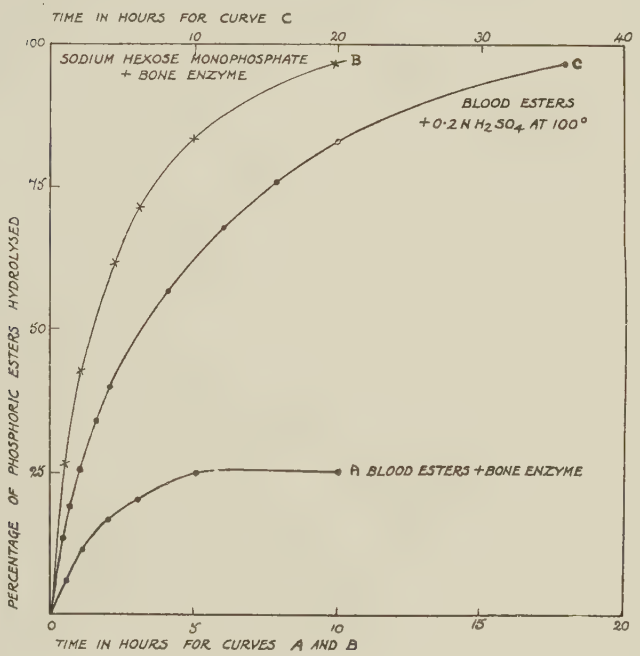


FIG. 16.—Hydrolysis of the organic acid-soluble phosphorus compounds of the blood by the bone enzyme and by sulphuric acid. (After Kay and Robison.)

that the phosphoric esters of the blood are divisible into two fractions, one of which represents that portion of the phosphorus compounds hydrolysable by the bone esterase, whilst the other portion is resistant to the action of this enzyme. Following up this information, Goodwin and Robison have prepared from deproteinised blood filtrates

two fractions of the barium salts of the phosphoric esters with the following properties :—

Fraction A.—Practically insoluble in water, no reducing action on Fehling's solution, forms the greater part of the total organic phosphoric acid, and contains the esters not hydrolysed by the bone enzyme.

Fraction B.—Readily soluble in water, reduces Fehling's solution, and is hydrolysed by the bone enzyme to the extent of 90 per cent. in six hours. The ratio of the reducing power calculated as glucose to the phosphorus content is very similar to that of hexosemonophosphoric ester. Fraction B is optically active, being *lævo*-rotatory.

One may therefore conclude that a portion of the phosphoric esters present in blood is rapidly hydrolysed by the bone enzyme, whilst the other portion is resistant to its action. Kay and Robison have been able to show that the greater part of the blood esters which are attacked by the bone enzyme is not hydrolysed by the phosphoric esterase of the muscle, and hence this portion of the esters constitutes a specific substrate for the bone enzyme such as is required for the scheme of ossification suggested by Robison and his co-workers. It will be seen from the table given on p. 194 that most of the acid-soluble organic phosphorus of the blood is present in the corpuscles. On the other hand it seems probable that ossification must be determined by the concentrations of calcium and phosphate ions in the plasma. Robison points out, however, that a very small amount of ester present in plasma saturated with calcium phosphate would be sufficient to bring about deposition of the solid phase by increasing the concentration of phosphate ions when hydrolysis occurred. On this view, if one regards the concentrations of ester in the corpuscles and plasma as being in equilibrium, the corpuscles would act as reservoirs from which the supply in the plasma would constantly be replenished.

The Significance of Phosphoric Esterase in the Kidney

It will be observed from the foregoing that a monophosphoric esterase is present in the kidney in amount and activity scarcely inferior to that of ossifying tissues. It is possible that its presence there may have some special significance in relation to the excretion of phosphates. Eichholtz and Starling have shown that the isolated kidney perfused by means of the heart-lung preparation will not concentrate the normal inorganic phosphate of the blood serum. On the other hand, Eichholtz, Robison, and Brull have shown that such a preparation is able to hydrolyse and to concentrate added organic phosphates and to excrete their phosphorus as inorganic phosphate in a much higher concentration than that of the inorganic phosphates of the serum. In the intact animal it is possible by removal of the pituitary body or by puncture of the *tuber cinereum* to cause the urinary inorganic phosphates to sink to a minimum. If now organic phosphate be injected, phosphorus is excreted, after an initial period, entirely in the inorganic form. The suggestion is put forward that normally a considerable part, if not all, of the urinary phosphates is derived from the organic phosphate of the blood serum. The hydrolysis of the organic blood esters is brought about in the cells of the kidney by the phosphoric esterase present there.

The Phosphatides

The phosphatides are organic phosphorus compounds (frequently referred to as lipins or lipoids) with a wide distribution in both animal and plant tissues. They are esters of phosphoric acid and possess chemical structures, and certain physical properties, analogous to those of the fats. They may be extracted along with the latter by the usual fat solvents. Their isolation and separation from one another in a pure condition is a matter of considerable difficulty, and it is probable that no other similar field of

biochemistry contains a more extensive graveyard of deceased compounds, with accompanying theories of their biological significance, than does this field. Of the phosphatides one may sing with the poet :

> " The dead are more in muster
> . . . than the quick,"

for at the present time the number of definitely characterised phosphatides may be limited to three—or probably four—although these may exist in numerous isomeric or closely related forms. They are the monoaminomonophosphatides lecithin and kephalin, the diaminomonophosphatide sphingomyelin, and a nitrogen-free phosphatide recently isolated from a vegetable source by Chibnall and Channon.

The Isolation of Phosphatides.—For the separation of phosphatides from tissues it is best to start with material which has been freed from visible fat and dried as rapidly as possible at a low temperature out of contact with air, otherwise there is no certainty that one will isolate the unchanged phosphatides. Drying the tissue with cold acetone is a very useful method, since most of the fat and cholesterol is removed along with the water, and at the same time very little phosphatide is lost. Drying at low temperature in a vacuum is also an effective method. Thereafter the selection of a suitable solvent must be determined by the particular phosphatide required. Levene gives the following generalisations : When it is desired to obtain very pure lecithin or pure kephalin the acetone extract is employed. When the object of the extraction is the preparation of all unsaturated phosphatides, then ether is chosen as the reagent. Finally, when it is desired to prepare sphingomyelin, the dried tissues are extracted directly by means of alcohol. As a matter of routine, all three reagents are employed consecutively, the procedure being as follows. The dry material is pulverised and extracted consecutively with acetone, ether, and alcohol. Each of the extracts is worked over separately. The acetone extract contains lecithin and kephalin and only a small

proportion of their decomposition products. This fraction is the one best suited for the preparation of pure lecithin. The crude ethereal extract contains unchanged lecithin and kephalin associated with the products of their partial decomposition. This fraction can be separated into two subfractions. One is soluble in cold as well as in hot alcohol, and is commonly named "lecithin"; and the other is soluble in ether but insoluble in alcohol, and is generally referred to as "kephalin."

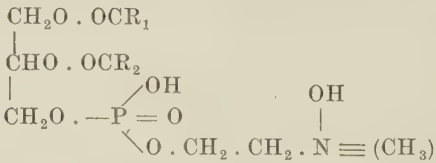
Maclean's general principles for separating phosphatides from associated substances are as follows: After thorough extraction of the tissue, the extract is concentrated and the residue treated with ether, when a thick opalescent mixture is obtained. The insoluble fraction consists partly of impurities, such as inorganic salts and nitrogenous substances, and partly of sphingomyelin and cerebrosides. This ether-insoluble part is separated either by filtration or by centrifuging, leaving a clear yellowish ether solution. This solution is concentrated and treated with excess of acetone, which precipitates the phosphatides, leaving cholesterol and fats in solution. On redissolving the precipitate in ether, a slightly opalescent solution is again obtained; the opalescence is due to the presence of small amounts of a mixture of sphingomyelin and cerebrosides. On centrifuging, the liquid yields a clear solution, which is concentrated and precipitated with acetone as before. The precipitated phosphatides are now comparatively free from cholesterol, fat, fatty acid, and sphingomyelin; by several repetitions of the above process of dissolving in ether and precipitating with acetone every trace of these impurities is eventually eliminated. The phosphatide mixture isolated in this way is dissolved in a small amount of ether and excess of absolute alcohol added. This addition of alcohol causes some of the phosphatide to separate; this alcohol-insoluble part constitutes crude kephalin. The alcohol-soluble part, when further purified, yields a substance with a nitrogen and phosphorus percentage

corresponding to that of lecithin and is considered to be pure.

These methods represent the essential manipulations, but many modifications may be introduced to suit particular cases. The cadmium chloride double compound of lecithin, which is crystalline and may be recrystallised from a mixture of alcohol and ethyl acetate, is very useful for the preparation of this specific phosphatide. In identifying phosphatides Levene has used extensively the catalytic hydrogenation method. The resulting saturated phosphatides are quite stable in air, are crystalline, readily recrystallised, and are consequently obtainable in a high state of purity.[1]

Structure.—The structure of each of the three phosphatides will be considered in turn.

(1) *Lecithin*, which on complete hydrolysis gives rise to the base choline, glycerol, phosphoric acid and fatty acids, is represented by the formula given, in which R_1 and R_2



represent the fatty acyl radicles. The lecithin obtained from various sources yields both saturated and unsaturated fatty acids, but the proportion of highly unsaturated acid is much higher in liver lecithin than in that obtained from egg-yolk or brain. The saturated acids are palmitic and stearic, and the unsaturated acids are oleic ($C_{18}H_{34}O_2$) with one double bond, linolic ($C_{18}H_{32}O_2$) with two double bonds, linolenic ($C_{18}H_{30}O_2$) with three double bonds, and finally arachidonic ($C_{20}H_{32}O_2$) with four double bonds. As yet all these un-

[1] For detailed methods of purifying phosphatides the original papers of Levene and of Maclean, or Maclean's monograph, should be consulted. Levene and Rolf have recently published useful accounts of the preparation and purification of lecithin and kephalin embodying all recent improvements in the methods used.

saturated acids have not been demonstrated in all the lecithins obtained from individual tissues, but they are probably all present nevertheless. Thus it appears that no less than six fatty acids have to be accommodated in the structure of lecithin. This, in the opinion of Levene (who with Maclean is responsible for most of the recent detailed structural investigations on phosphatides), can best be achieved by the assumption that different lecithins exist, the variations being in the nature of the fatty acid residues. Thus stearyl-oleyl, palmityl-oleyl, stearyl-linolenyl, and stearyl-arachidonyl lecithins are all possible and probably do occur.

Lecithin from a vegetable source (soya-bean) contains less saturated fatty acid than lecithin from animal tissues, but again palmitic and stearic acids are found in the saturated fraction, and oleic, linolic, and linolenic acids occur in the unsaturated fraction. In the latter unsaturated hydroxy fatty acids are probably also present.

There is a further possibility which must be considered in relation to the problem of the structure of lecithin. It is possible by mild alkaline hydrolysis to isolate the glycerophosphoric acid of lecithin, and this proves to be optically active. Hence of the two possible isomers of glycerophosphoric acid, the α-form, $CH_2OH \cdot CHOH \cdot CH_2OP$, and the β-form, $CH_2OH \cdot CHOP \cdot CH_2OH$ (in which P represents the phosphoric acid residue), the latter would appear to be excluded, since it does not possess an asymmetric carbon atom. The existence of a lecithin derived from the first of these isomers is undisputed, nevertheless it has been claimed by Bailly that it is possible to isolate from the glycerophosphoric acid of lecithin two salts of different crystalline form, one of which yields dihydroxyacetone monophosphate on oxidation with bromine, whilst the other fails to do so. To the latter the β-structure, $CH_2OH \cdot CHOP \cdot CH_2OH$, is ascribed. This observation further multiplies the possibilities of isomerism in the lecithins. Lecithin itself is optically

active in a *dextro*-rotatory sense, but both types of glycerophosphoric acid can give rise to asymmetric lecithins.

$$CH_2OR_1 . CHOR_1 . CH_2OP \quad . \ . \ . \ . \ . \quad (1)$$
$$CH_2OR_1 . CHOR_2 . CH_2OP \quad . \ . \ . \ . \ . \quad (2)$$
$$CH_2OR_1 . CHOP . CH_2OR_2 \quad . \ . \ . \ . \ . \quad (3)$$
$$CH_2OR_1 . CHOP . CH_2OR_1 \quad . \ . \ . \ . \ . \quad (4)$$

Of the formulæ given above (1) and (2), derived from the α-glycerophosphoric acid, and (3), derived from the β-acid, would be optically active, whilst only (4) would be inactive. (R_1 and R_2 represent the fatty acid residues.)

(2) *Kephalin* yields on hydrolysis a series of compounds very similar to those obtained from lecithin ; in fact the only essential difference is the presence of the much weaker base aminoethyl alcohol, $CH_2OH . CH_2NH_2$, in place of choline, so that with this difference the formula already given for lecithin represents also that of kephalin. Questions already discussed relating to the structure of lecithin therefore apply to the constitution of kephalin. It must, however, be pointed out that it is much more difficult to obtain the fatty acids of kephalin free from phosphoric acid than it is in the case of lecithin. On this account Maclean has considered the possibility of Thudicum's original suggestion being correct, namely, that the arrangement : Fatty Acids—Glycerol—Phosphoric Acid found in lecithin is modified in kephalin to : Glycerol—Phosphoric Acid—Fatty Acids. The isolation from kephalin of definite intermediate compounds of fatty acid and phosphoric acid has been claimed by Thudicum and by Parnas. The bulk of the evidence seems nevertheless to favour the view that lecithin and kephalin are constituted on analogous lines. The fatty acids of kephalin have not been so extensively investigated as have those of lecithin, but they appear to be the same. Thus the unsaturated acids definitely known to occur in kephalin are oleic, arachidonic (in brain kephalin), and linolic acids. Of the saturated acids stearic only has so far been detected. Kephalin of vegetable

origin (soya-bean) shows no marked difference from that of animal origin.

Lysolecithin (Lysocithin) and Lysokephalin.—Delezenne and Fourneau showed that by acting on lecithin of egg-yolk with snake (cobra) venom a partial hydrolysis occurred, involving the removal of unsaturated fatty acids, and a crystalline compound was obtained which had the composition of a choline monopalmitylglycerophosphate. They called this compound lysocithin. The action of the venom is due to the presence of an enzyme which has the remarkably specific property of hydrolysing only the unsaturated fatty acid groups. Levene and his associates have more recently shown that kephalin undergoes a similar loss of its unsaturated fatty acids when acted on by cobra venom. They suggest the names lysolecithin and lysokephalin for these compounds, and in as far as lecithins differing in the nature of their saturated fatty acids are known to occur, it is probable that at least two of these lysolecithins exist. These interesting compounds shed considerable light on the question of the structure of the parent phosphatides. From the mixture of lysolecithin and lysokephalin prepared from egg-yolk two fractions are obtained, one yielding palmitic and stearic acids (from lysolecithin), and the other stearic acid only (from lysokephalin), on hydrolysis. Thus the finding of stearic only in the saturated acids of kephalin is paralleled by its presence alone in lysokephalin, whilst both the saturated acids of lecithin turn up in the lyso compound. Further, if egg-yolk contained lecithins derived from saturated acids only, these would survive the action of the venom, and if dioleyl lecithins were present one would expect to find an oleyl lysolecithin (or choline glycerophosphate) in the product containing the lyso compounds. But no unchanged lecithin is found to be present, nor are unsaturated lysolecithins known, so that the conclusion that only mixed lecithins occur in nature, that is, those containing a saturated and an unsaturated fatty acid residue, would seem to be

justified. These partially hydrolysed phosphatides exert a powerful hæmolytic action on red blood cells, lysolecithin being three times as active as lysokephalin in this respect. It is probable that the hæmolytic properties acquired by blood serum to which a small quantity of snake venom has been added are due to the conversion of the serum phosphatides to the corresponding lyso compounds, which are also toxic in themselves. The lyso phosphatides have a marked affinity for cholesterol, the loose compound formed consisting of monomolecular proportions of each component. It is devoid of hæmolytic properties. Lysolecithins have been used for the preparation of synthetic lecithins ; thus acetyl-, benzoyl-, oleyl-, and elaidyl-lecithins have been obtained by Levene and Rolf in a pure state by acting on lysolecithin with the appropriate acid chloride. They have no hæmolytic action, and most of them form waxy masses ; oleyl-lecithin so prepared is, as one might expect, closely similar to natural lecithin. The preparation of synthetic lecithins and kephalins from $\alpha\beta$- and $\alpha\gamma$-distearins has been recently described by Grün and Limpächer.

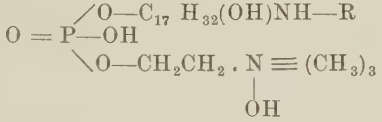
(3) *Sphingomyelin* yields on hydrolysis four components in equivalent proportions. These are phosphoric acid, two bases, choline and sphingosine, and fatty acids. It is therefore a diaminomonophosphatide. Of the bases choline is much more easily separated by hydrolysis than is sphingosine. The occurrence of sphingosine (and therefore of sphingomyelin) is limited to the animal phosphatides. The structure of sphingosine is

$$CH_3 . (CH_2)_{11} . CH = CH . CHOH . CHOH . CH_2NH_2,$$

but the allocation of the amino group and the two hydroxyl groups between carbon atoms 1, 2 or 3 is not yet established, nor is the stereochemical configuration of the

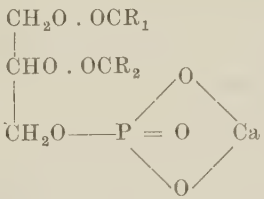
CHOH groups. Two fatty acids have been obtained up to the present time from sphingomyelin, and these are both

saturated—lignoceric acid, $C_{24}H_{48}O_2$, and a hydroxy derivative of an acid of lower carbon content, which is possibly hydroxystearic acid. On the analogy of the other phosphatides it is reasonable to assign the central position in the structure of this phosphatide to the hydroxy compound, in this case the base sphingosine. Neither sphingomyelin nor lignocerylsphingosine obtained from it on partial hydrolysis contain a free primary amino group. These facts together with the ease of detachment of the choline residue are best accommodated by assigning to the phosphatide the structure—



in which R represents the fatty acid. As in the case of lecithin and kephalin, one must suppose that at least two sphingomyelins exist differing from one another in the nature of their fatty acids.

(4) The nitrogen-free phosphatide recently isolated by Chibnall and Channon has been shown by its discoverers to have the following constitution, R_1 and R_2 representing, as in the case of lecithin, fatty acid residues, one of which at least is unsaturated :—



The most noteworthy feature of this new phosphatide is the presence of calcium, and it provides, so far as the writer is aware, the first recorded instance of an ether-soluble calcium derivative occurring in Nature. Chibnall and

Channon obtained the phosphatide by precipitating with acetone an ether extract of pressed and dried green leaves of unheaded cabbage, *Brassica oleracea*. In 1909 Winterstein and Stegmann recorded the presence of both phosphorus and calcium in the ether-soluble material obtained from dried *Ricinus* leaves, but these earlier workers did not attempt to isolate the calcium-containing compound. Chibnall and Channon have given the name " phosphatidic acid " to the parent substance of which the new phosphatide is the calcium salt, thus emphasising its relationship to the nitrogenous phosphatides lecithin and kephalin. The fatty acids present in the new phosphatide are stearic, palmitic, linolic, and linolenic. The unsaturated acids are, like those of the soya bean phosphatides and unlike those of the phosphatides of animal origin, in excess of the saturated acids. In the course of isolation of calcium phosphatidate, Chibnall and Channon obtained from some 220 kilograms of cabbage leaves 1·1 gram of a substance containing phosphorus, but no nitrogen, and 8·9 per cent. of iron. This interesting iron compound is freely soluble in ether, but does not appear to be of the nature of a phosphatide.

The numerous phosphatides whose existence has been reported from time to time, such as heparphosphatide, cuorin, protagon, tethelin, carnaubon, vesalthin, neottin, sahidin, jecorin, etc., in which various N : P ratios are reported, must be regarded as mixtures of the three known phosphatides with each other, or with their degradation products, or with the cerebrosides which have solubilities and distributions similar to those of the phosphatides.

Phosphatides in Metabolism. -We have already discussed in Chapter VI. those aspects of the metabolism of phosphatides which are correlated with the utilisation of fat in the organism. Turning now to the more specialised aspects of this problem, we have to note first of all that there is little information relating to the mode of origin of phosphatides in either animal or plant tissues. There is in the intestinal

mucosa an enzyme capable of hydrolysing (and presumably therefore of synthesising) lecithin, and the occurrence of such an enzyme in takadiastase—that storehouse of enzymes—has also been recorded. Again, it is claimed that hens or ducks kept on a diet with a minimal lecithin content lay eggs with a normal content of the phosphatide, so that the parent bird appears to be able to synthesise the latter. This is supported by the observation of McCollum and his co-workers that the nature of the fatty acids of lecithin in the egg-yolk changes with the nature of the diet, as is shown in the following table (from Maclean, "Lecithin and Allied Substances," 2nd ed., p. 175) :—

TABLE XV

Diet.	Iodine number of:					
	(*a*) Fats.			(*b*) Phosphatides.		
	(1)	(2)	(3)	(1)	(2)	(3)
Ration nearly free from fats and phosphatides .	50·0	54·36	51·1	35·22	34·07	34·0
Normal ration .	63·2	65·5	—	63·7	63·1	

It is therefore clear that the degree of saturation of the fatty acids of the phosphatides is even more affected than is that of the fats by a variation in the diet. Eichholtz has recently reported, however, that lecithin, when fed to young dogs, increases the ether-soluble phosphorus of the blood, but not the total fat, and he concludes that lecithin is assimilated as such and that its fatty acid residues are not built up into neutral fats, which process would necessitate a breakdown of the lecithin fed. Eckstein has, however, arrived at a diametrically opposite conclusion. He reports that the ingestion of lecithin increases the total fatty acid content of the lymph, and concludes that, like simple fats, lecithin is

transformed into neutral fat during its transport through the intestinal mucosa.

The association of fats with phosphoric acid which we encounter in the phosphatides is highly suggestive now that we realise the importance of phosphoric acid compounds in the intermediary metabolism of the carbohydrates. Indeed, as we have seen in an earlier chapter, where the evidence is reviewed, theories have been advanced which postulate that fatty acids, before oxidation, are combined with glycerophosphoric acid. It is known that the neutral fats of the liver are more highly unsaturated than the fats of other tissues, and Levene's demonstration that the fatty acids of the liver phosphatides are likewise more unsaturated than those of brain and egg-yolk phosphatides is in harmony with the general theory mentioned.

A study of the phosphorus metabolism of the developing egg during incubation elucidates the catabolism of the phosphatides rather than their anabolism. Thus it will be seen from the accompanying curves in Fig. 17, drawn from the data of Plimmer and Scott, that the steady drop in phosphatide phosphorus, which represents some 65 per cent. of the total phosphorus in the unincubated egg, and in the phosphoprotein phosphorus during incubation, is accompanied by an equally steady increase in inorganic phosphate and in nucleoprotein phosphorus ; the large amounts of phosphatide in the hen's egg appear to furnish a store of phosphoric acid, which is released as it is required for other purposes, *e.g.*, the synthesis of the important nucleoproteins, and for ossification. In regard to this last question it is very interesting to note that Kay (quoted by Needham) reports the absence in the unincubated egg of both the bone enzyme of Robison and of a suitable substrate, a finding which is in agreement with the low content of acid-soluble organic phosphorus found in the early stages of incubation of the egg by Plimmer and Scott. By the twelfth day of incubation Kay finds both the enzyme and substrate in considerable amount. From this

time onwards, however, as will be seen from the curve, the acid-soluble organic phosphorus of the embryo chick *only* steadily and rapidly diminishes (a change which is also shown, but less strikingly, by the whole egg), and this period coincides with vigorous bone development. These facts lend further

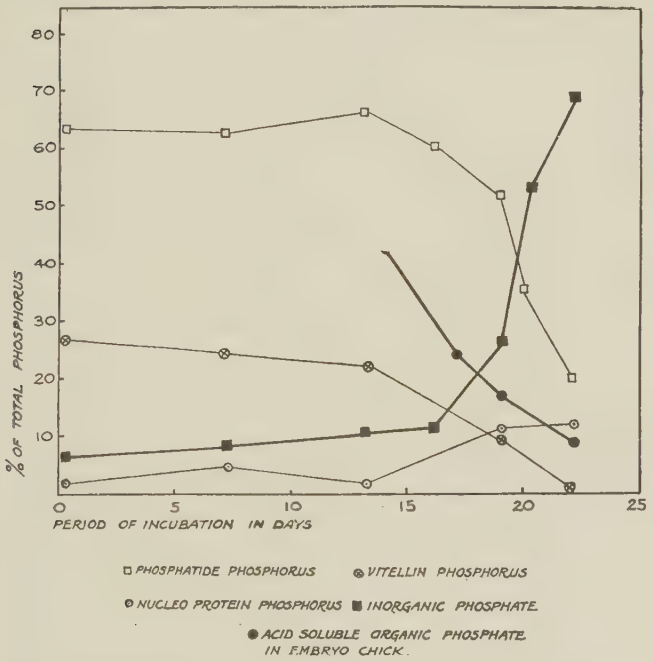


FIG. 17.—Alterations in the distribution of phosphorus in the incubating egg. (Drawn from data of Plimmer and Scott.)

striking support to the views of Robison on ossification, and in this particular case it is probable that a considerable proportion of the phosphoric acid released from the phosphatides goes to form hexose or similar phosphates, which are then broken down and their phosphoric acid eventually deposited in bone as calcium phosphate.

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CHAPTER VIII

THE RÔLE OF SULPHUR IN BIOCHEMISTRY

Sulphur is a universal but quantitatively minute component of practically all animal tissues. It finds its way into the animal body chiefly in the form of the amino acids cysteine and cystine, derived from proteins, and to a much less extent in phosphatides containing sulphur, and in inorganic sulphates, etc.

There is a considerable amount of evidence pointing to the presence in the protein molecule of a source of organic sulphur additional to cysteine or cystine. Thus casein which yields only traces of cystine loses but 13 per cent. of its total sulphur on boiling with alkali, and ovalbumin gives a very similar result. The amount of methyl sulphonic acid obtained from proteins on oxidation with nitric acid bears no relation to their cystine content, nor is cystine regarded as a possible source of methyl sulphonic acid in such oxidations. Mueller, indeed, has recently isolated a compound of the formula $C_5H_{11}O_2NS$, isomeric with ethyl cysteine, from various proteins, including casein. It is regarded as an α-amino acid and a primary product of protein cleavage. It gives no nitroprusside reaction, does not blacken lead, and on being fed to man its sulphur, which is more firmly bound than that of cysteine, is readily oxidised and excreted as inorganic sulphate. Another observation of importance in relation to this question has been made by Hoffman and Gortner. These workers prepared from pure crystalline cystine (hexagonal plates) an isomeric cystine (microscopic prisms) obtained by prolonged boiling of the former with hydrochloric acid. The new form of cystine is optically inactive and is more soluble in water than "plate" cystine, as also is its phosphotungstate more soluble than that of the natural "plate"

cystine. It is suggested that this isomeric cystine is the type obtained in artificial syntheses. In more recent experiments, Hoffman and Gortner have failed to resolve the new inactive form of cystine either by biological or chemical means. They suggest that it is probably an internally compensated form. The differences between *l*-cystine and *i*-cystine extend to their derivatives.

The fact that cysteine, $CH_2SH \cdot CHNH_2 \cdot COOH$, is one of the indispensable amino acids which the animal is unable to synthesise, and which must be supplied to it in some form or other with the protein portion of a dietary in order that the life processes may proceed normally, has led biochemists to attach to this sulphur-containing acid a peculiarly important *rôle* in animal metabolism.

Cysteine itself is a typical crystalline amino acid which may be obtained from all " complete " proteins on hydrolysis, and which is characterised by the ease with which it is transformed into its bimolecular form, cystine, with the loss of one atom of hydrogen from each of the two cysteine molecules. This conversion takes place in the presence of molecular oxygen and is greatly facilitated by a slight degree of alkalinity in the solution. Again, with a suitable choice of conditions it is possible to reverse this process and convert the oxidised cystine back again to the reduced cysteine, mild reducing agents effecting this change slowly, more vigorous reagents, such as tin and hydrochloric acid, rapidly.

$$O + \begin{matrix} COOH \cdot CHNH_2 \cdot CH_2SH \\ COOH \cdot CHNH_2 \cdot CH_2SH \end{matrix} \underset{+2H}{\overset{+O}{\rightleftharpoons}} \begin{matrix} COOH \cdot CHNH_2 \cdot CH_2{-}S \\ | \\ COOH \cdot CHNH_2 \cdot CH_2{-}S \end{matrix} + H_2O$$

It is obvious that such a reversible autoxidisable system as that pictured above is of great interest to biochemists, and Heffter in 1908 and Mathews and Walker in 1909 were amongst the first to suggest that cysteine or cystine, or a complex of one or both of these bodies with a protein or similar residue, is related in an intimate way to tissue

respiration, that is to say, with the oxidation and reduction processes which go on within the living cell, combined cysteine constituting as it were a *point d'appui* in the molecule around which these reactions are initiated. If such views could be substantiated the necessity for the inclusion of these sulphur-containing amino acids in any dietary would receive a ready explanation.

The Autoxidisability of Cysteine.—The problem of the autoxidisability of cysteine has therefore an obviously important bearing on the whole question of tissue oxidations, and has recently been the object of much investigation. Like most reactions of biochemical interest, it is found to be exceedingly susceptible to the influence of small amounts of foreign substances, and indeed Warburg and Sakuma regard the autoxidation of cysteine to cystine as a catalytic phenomenon dependent on the presence of traces of heavy metals, more especially of iron. This hypothesis affords a simple and convenient explanation of the marked inhibitory action of hydrocyanic acid and the cyanides generally on the autoxidation of cysteine, a phenomenon first recorded by Mathews and Walker, especially when one calls to mind the tendency of iron and the CN radicle to form complex ions in which the iron no longer carries a positive charge. In substantiation of these views Sakuma has prepared a specially pure cysteine which he recrystallised from ethyl or propyl alcohol, using quartz vessels for all operations, including the preparation and purification of reagents and solvents, and thus reducing possible contamination by iron and other heavy metals to a minimum. Such a preparation of cysteine shows a rate of autoxidation measured in quartz vessels, some 100 to 250 times less than that of previous "pure" preparations of the amino acid, and it is estimated that some 99·7 per cent. of the so-called autoxidisability of cysteine depends on a catalysis resulting from impurities. Working with highly purified preparations of this kind it is found that the merest trace of iron has a marked effect in catalysing the

rate of oxidation to cystine (see Fig. 18), and Warburg states that 1/100,000 mg. of iron in 10 c.c. is detectable by its effect in promoting this catalysis. Abderhalden and his co-workers, however, adhere to the view that cysteine is autoxidisable in the complete absence of iron, although their precautions to exclude possible traces of the metal do not seem to have been so rigorous as those of Warburg and Sakuma. In any case Abderhalden attaches importance to the slight residual oxygen uptake shown by the purest available preparations

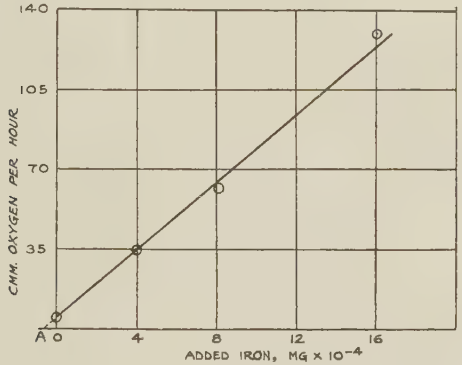


FIG. 18.—Effect of increasing concentrations of iron on the rate of oxygen uptake by pure cysteine hydrochloride (12 mg.). Iron added as $FeCl_3$; $p_H = 8{\cdot}0$; temp. 20·0°. (After Harrison.)

of the amino acid. Harrison, reinvestigating the question, has confirmed most of the observations of Warburg, but nevertheless finds that the slight residual autoxidisability is rather more than can be accounted for by the trace of iron still present in his preparations. Thus by extrapolation of his curve showing the linear relationship between oxygen uptake and amount of iron present (Fig. 18), the distance AO, which corresponds to a possible residual trace of iron necessary to produce the effect observed, is found to represent 0·00005 mg. iron in the 12 mg. of cysteine used. In the actual preparation the amount of iron found was only 0·00001 mg.,

from which Harrison concludes that the residual uptake of oxygen is not entirely due to the presence of iron. Hence either traces of catalysts other than iron are present in the purified cysteine, or cysteine is to a minute extent strictly autoxidisable. The latter conclusion would seem to correlate well with the classical definition of a catalyst as a substance which modifies the velocity of a reaction, but which cannot of itself initiate that reaction. The reaction must therefore be already in progress, however slowly, before it may be catalysed.

The Autoxidisable Sulphur Constituent of the Cell—Glutathione

Whilst the cysteine-cystine system (with appropriate catalysts) might, as we have seen, play an important part in tissue respiration, there is in actual fact no evidence to show that either free cysteine or cystine is present in normal tissues, and the low solubility of cystine at reactions in the vicinity of neutrality does not accord with the supposition that it is fitted to play such a part. Nevertheless the fact that most tissues which are the seat of active metabolic processes give the nitroprusside reaction, which we shall discuss later, has been accepted for some time as evidence of the occurrence in these tissues of some compound very closely related to cysteine. An examination of the constituents of extracts from such tissues initiated by Hopkins has led in recent years to the isolation of the compound responsible for the reaction, and to a very noteworthy extension of our knowledge of the autoxidisable systems of the living cell and of the oxidative and reductive changes associated with tissue respiration. Hopkins gave to his new substance the name *glutathione*, since he demonstrated it to be a dipeptide of cysteine and glutaminic acid. It is of very wide occurrence and was first isolated from yeast cells and from mammalian liver and muscle, in which it is found in a free uncombined state. A description of the method by which this substance may be isolated is given.

Isolation of Glutathione.—Yeast (45 kg.) is boiled with three successive quantities (each 10 litres) of tap water, filtered hot each time, and the combined extracts are partially neutralised with dilute sodium hydroxide. To the still slightly acid extracts neutral lead acetate is added, and the resulting precipitate is allowed to settle and separated from the supernatant solution by siphoning. The precipitate, after being thoroughly washed on large Büchner filters, is ground in mortars and extracted with cold n/2 sulphuric acid until fresh extracts cease to give the nitroprusside reaction. Uranium acetate is then added to the sulphuric acid extracts, which amount to about 12 litres, until it is present in slight excess. Hot saturated barium hydroxide solution is now added until precipitation ceases, and the precipitate is filtered under pressure and washed with cold water. These treatments remove various peptides and all the phosphoric acid from the yeast. From the filtrate barium is removed as the sulphate and acid mercuric sulphate is added. The precipitate is washed, suspended in water, treated with H_2S, and the filtrate and washings, after being freed from H_2S by aeration, are made half normal in acidity with sulphuric acid. The solution (1,000–1,500 c.c.) is then treated with phosphotungstic acid until the precipitate is maximal, the latter is filtered, and from the filtrate the excess precipitant is removed by barium hydroxide, and the latter by sulphuric acid. The solution is once again treated with acid mercuric sulphate, the suspended mercury precipitate is decomposed with H_2S, and the sulphide precipitate washed with small volumes of water to keep the concentration of the product as high as possible. From this solution, now rich in glutathione, the dipeptide may be separated either as the copper or as the lead compound.

Copper Compound.—To the solution obtained from the second mercury separation, moist, freshly precipitated copper hydroxide is added, followed by sufficient sodium hydroxide to render the supernatant blue liquid nearly

neutral to litmus. The copper compound so obtained is decomposed, after separation, with H_2S, and after removal of excess of the latter the filtrate is made alkaline with barium hydroxide and aerated to ensure oxidation of the dipeptide to the bimolecular form, using the nitroprusside test, which is only given by the reduced form, as a control. The solution is then carefully freed from both barium and sulphuric acid, concentrated under reduced pressure to about 10 c.c., and poured into 100 c.c. of absolute alcohol. The precipitated glutathione should then stand under the alcohol until it is quite dry and friable.

Lead Compound.—Lead acetate is added to the solution resulting from the decomposition of the second mercury precipitate, and the resulting lead salt is decomposed with H_2S. The subsequent precedure is then the same as that described for the copper compound.

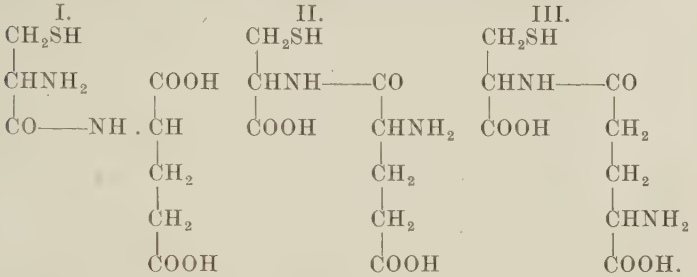
Working on a small scale the silver compound, obtained by treatment with silver sulphate and barium hydroxide, may conveniently be used for purposes of purification. It is soluble in hot water and separates again on cooling.

In extracting animal tissues the finely minced organ is allowed to extract twice for two to three hours with its own weight of water at 40°, and the procedure is then as described for yeast.

The oxidised form of glutathione is a snow-white, non-hygroscopic powder, which softens at 165° to 167° and melts with decomposition at 182° to 185°. It is readily reduced to the mono-molecular compound, which may be obtained in a crystalline form.

The Constitution of Glutathione.—The elucidation of the constitution of glutathione provides an admirable illustration of the application of analytical and synthetical methods to the complete solution of a none too easy problem, rendered much more difficult by the small scale on which it was necessary to work owing to the difficulty of securing adequate supplies of the substance. It is worthy of consideration in some detail.

As has been mentioned, Hopkins showed glutathione to be a dipeptide of glutaminic acid and cysteine of the formula $C_8H_{14}O_5N_2S$. Such a dipeptide in its mono-molecular form may possess any one of the following three formulæ :—



The facts on which a decision may be made have been presented in a paper by Quastel, Stewart, and Tunnicliffe. These are :—

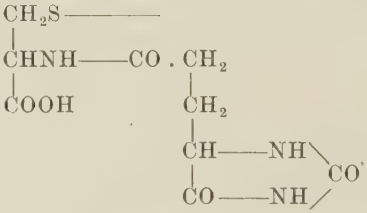
(1) Glutathione, after being condensed with 2 : 3 : 4 trinitrotoluene, which reacts with primary amino groups, and then subjected to hydrolysis, yields free cysteine.

(2) On replacing the free amino group of glutathione with hydroxyl, using ice-cold hydrochloric acid and silver nitrite, and on hydrolysing the product, α-hydroxyglutaric acid is obtained.

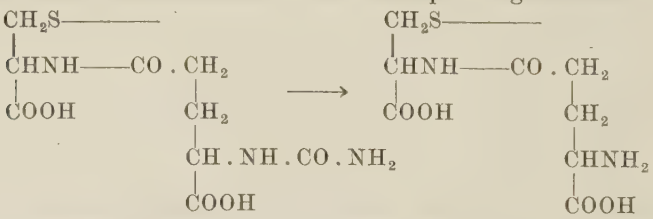
(3) On oxidising glutathione with hydrogen peroxide in the presence of a trace of ferrous sulphate, a reaction which converts α-amino acids into carboxylic acids with one carbon atom less, and on hydrolysing the oxidation product, free succinic acid is obtained.

Results (1) and (2) exclude formula I. as being that of glutathione, since in it the free amino group which would condense with trinitrotoluene is in the cysteine residue, and hence free cysteine would not be obtained on hydrolysis ; whilst likewise α-hydroxyglutaric acid could not be formed on deaminising and hydrolysing a peptide of this constitution. Of the remaining two formulæ only a compound of the constitution III. could give the third result obtained, since

even if the $>CH \cdot NH_2$ group of compound II. were oxidised, this would of necessity involve a break in the chain and succinic acid would appear *before* hydrolysis if it were to be formed at all. Thus the analytical findings point to glutathione having the constitution III. This has been fully confirmed by Stewart and Tunnicliffe, who succeeded in synthesising compound III. and established its identity with natural glutathione. The series of reactions employed was : the conversion of glutaminic acid into hydantoinpropionic acid, the preparation of the acid bromide of this latter acid, and its coupling with cystine dimethyl ester hydrochloride, whereby the compound :



dihydantoinpropionyl cystine was obtained. By boiling with calcium hydroxide solution, followed by treatment with nitrous acid, the hydantoin ring was opened, and the resulting uramino acid was converted to the corresponding amino acid :



This compound proved to be identical with naturally occurring glutathione except for the fact that, whilst the latter has in aqueous solution the specific optical rotation $[\alpha]_{5461}$ — 98·3°, the synthetic product showed only $[\alpha]_{5461}$ — 30·6°, the discrepancy being ascribed to racemisation during the formation and subsequent opening of the hydantoin ring.

However, by an alternative synthesis affording further confirmatory evidence of constitution, a preparation has been obtained possessing the same optical activity as the natural dipeptide. On treating glutaminic acid with phosphorus tribromide in dry toluene the mono-acid bromide, $BrOC . CH_2 . CH_2 . CHNH_2 . COOH$, was obtained, and this on condensation with cystine dimethyl ester yielded glutathione possessing the specific rotation $[\alpha]_{5461} - 97{\cdot}4°$, in very close agreement with the constant for the natural product. We may therefore accept this work of the Cambridge laboratory as establishing the constitution of this interesting dipeptide. It should be noted that these syntheses yielded the oxidised or bimolecular form of the dipeptide.

Occurrence, Distribution, and Determination of Glutathione. —In his early work on yeast Hopkins obtained from 0·1 to 0·15 gram of glutathione in each kilogram of material extracted, a yield which was increased in later experiments. More recently Tunnicliffe, working in the Cambridge laboratories, has devised a method of determining glutathione and has made a systematic investigation of the distribution of the substance in various cells and tissues. The quantitative determination of glutathione is carried out as follows. A known weight of the tissue is ground in a mortar with sand and 10 per cent. trichloracetic acid, and after filtration under pressure it is re-extracted twice with the 10 per cent. acid. The clear extracts are combined and titrated with n/100 iodine, using sodium nitroprusside as an external indicator. This substance gives a purple colour with the reduced, but not with the oxidised, form of the dipeptide. From the equation :

$$2\,G . SH + I_2 \longrightarrow G . SS . G + 2\,HI,$$

1 c.c. of n/100 iodine corresponds to 2·5 mg. of reduced glutathione. The method gives satisfactory results when known amounts of glutathione are added to tissue pulp, and no abnormal results are obtained when urea, uric acid,

creatinine, glucose, or fructose are present. Determinations in tissue extracts of the soluble organic sulphur by difference (total sulphur *minus* sulphate sulphur) give results in close accordance with the iodine titration figure, from which it is concluded that in the latter method the sulphydryl groups alone are being estimated, and that by far the greater part of the glutathione is present in the tissues in the reduced form. Using the iodine titration method, and assuming that the soluble sulphydryl groups represent glutathione only, the following average percentage distributions of the dipeptide are given :—

Fresh yeast	0·18
Rat liver (glycogen free)	0·18
Rabbit liver (glycogen free)	0·24
Rat skeletal muscle	0·034
Rabbit skeletal muscle	0·045
Kidney	0·17
Blood	Absent.
Hen's egg	Absent.

The assumption that one is dealing with glutathione only in the above determinations is justifiable until it can be shown that other peptides containing cysteine of the type of glutathione do occur in tissues.

The Autoxidisability of Glutathione

We have seen that the autoxidation of cysteine is a reaction which proceeds of itself very slowly, but that it may be catalysed by the presence of traces of iron. Harrison has shown that glutathione is essentially analogous in its behaviour. When it is purified with the same precautions as were used in the case of cysteine, it is found that the rate of uptake of oxygen is greatly lowered, that the presence of minute amounts of iron markedly increase the rate of oxidation, and that this process is inhibited by the addition of hydrocyanic acid, the relative amount of inhibition being less in samples containing small amounts of iron than in

those containing more iron. These results are shown in Fig. 19, from which it will be seen that the rate of oxygen uptake by impure glutathione is very rapid, being practically complete in half an hour (curve D), whereas with purified glutathione the process is scarcely complete in six hours (curve B). Curve A shows the enormous accelerating effect

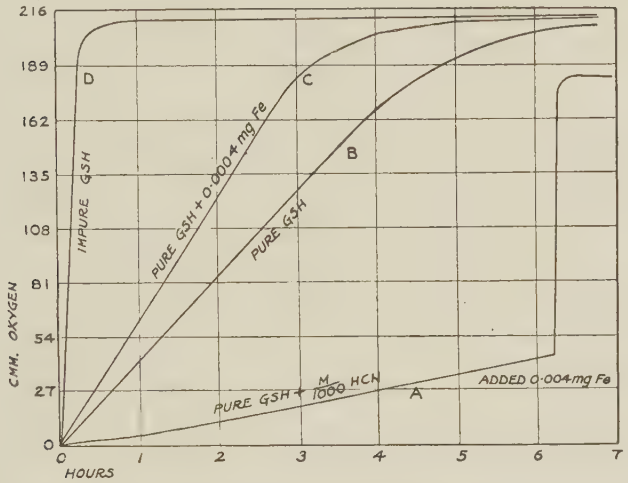


Fig. 19.—Comparison of the rates of oxygen uptake in the following systems:

A. 16 mg. pure GSH + $\frac{M}{1,000}$ HCN.
B. 16 mg. pure GSH.
C. 16 mg. pure GSH + 0·0004 mg. Fe (as $FeCl_3$).
D. 16 mg. crude GSH.
p_H = 8·0; temp. = 20°.

(After Harrison.)

of a minute trace (0·004 mg.) of iron on a sample of pure glutathione which was oxidising very slowly owing to the presence of cyanide. An inhibiting effect very similar to that produced by cyanide may be brought about by the addition of sodium pyrophosphate, which, like cyanide, is known to form complexes with iron. The difference between the autoxidation of cysteine and that of glutathione is therefore

one of degree rather than kind, and whilst in the cases of aerobic oxidation which we have been discussing here the dipeptide oxidises more rapidly than the amino acid, the reverse is the case in anaerobic oxidation, when instead of oxygen the dyestuff methylene blue is used as the hydrogen acceptor. More recently Harrison has shown that iron also catalyses the anaerobic oxidation of glutathione in the presence of methylene blue as hydrogen acceptor. This reaction, like the aerobic oxidation, is inhibited by cyanide. Copper may take the place of iron in such reactions. These facts all point to the necessity for some interaction between iron, or some similar metallic catalyst, and glutathione before the latter can exhibit the characteristic autoxidisability which it possesses in the tissues, and the universal occurrence of iron in the mammalian and most other tissues ensures that an adequate supply of this catalyst will be forthcoming. Thus it would seem that extremely minute traces of iron and other metals may play important parts in biological oxidations involving the sulphydryl group.

The Nitroprusside Reaction.—As has been mentioned, the nitroprusside reaction is used by biochemists as a specific test for organic sulphydryl groups in tissues and tissue extracts. As applied to these latter, it was first used by Arnold. The test is best carried out as follows: To the tissue extract, or to the fluid in which a piece of the fresh tissue is suspended, are added a few cubic centimetres of a saturated solution of ammonium sulphate followed by two or three drops of a 5 per cent. solution of sodium nitroprusside and then excess of ammonium hydroxide. A positive reaction is indicated by the development of an intense purple colour very like that of permanganate. The colour is intensified in the case of fresh tissues by a preliminary heating with very dilute acetic acid, or by grinding the tissue with sand. It will be seen that the mechanism of the reaction must be very similar to that of the Rothera test for acetone.

The substance which gives the nitroprusside reaction, and

which we may assume with considerable confidence to be glutathione, may be extracted from the tissues by hot water, by washing with isotonic solutions containing acetic acid, or even by prolonged washing with tap water. The applicability of the reaction is illustrated by the following cases. We have seen that the method available for the quantitative determination of glutathione reveals the absence of the latter from the hen's egg, and this is confirmed by the negative nitroprusside test. But after incubating the egg for thirty hours, by which time development of the embryo with incipient oxidation processes has begun, a strong nitroprusside reaction is observed. Again, Shearer has demonstrated that in the immature eggs and sperm of echinoderms the nitroprusside reaction is negative. It is positive in the ripe eggs and sperm, and one minute after fertilisation with an increase in oxygen consumption estimated at 8,000 per cent. of that of the unfertilised egg it has become very intense. Abderhalden and Wertheimer have shown that in the tissues of pigeons kept on a deficient diet of polished rice the nitroprusside reaction is very weak although cystine is apparently present in normal amounts. The pigeons exhibit a very much impaired gaseous metabolism, with diminished cell respiration and a low body temperature. The vitamin-deficient diet appears here to have produced an inability to convert cystine into cysteine, in which form it gives the nitroprusside test, with a consequent lowering of respiratory efficiency. A similar absence of nitroprusside reaction was demonstrated in the tissues of rats in which a failure of nutrition was produced by a deficiency of cystine in the diet. Abderhalden attempts to correlate these observations by suggesting that in alimentary dystrophia (vitamin deficiency) the normally reversible reaction, R—SH + HS—R $\rightleftharpoons$ R—S . S—R, can proceed only towards the right with the formation of the oxidised form of glutathione, so that the cell loses its powers of reduction. It should be added that these observations of Abderhalden have been challenged by other

workers, and, in any case, certain of his results may be due simply to starvation and not necessarily to vitamin deficiency.

It is not proposed to discuss here the part played by glutathione in promoting tissue respiration, as the reader will find an admirable account of the subject in the companion volume of the present work, Professor Lovatt Evans' "Recent Advances in Physiology." Suffice it to state here that the views now held concerning the *rôle* of glutathione as a co-enzyme of the oxidative-reductive systems of the tissues are firmly grounded on numerous observations, and these views afford an explanation of the reason why the amino acid cysteine is essential for the growth of young animals and for the preservation of nitrogenous equilibrium in the adult, an explanation which is at least as satisfactory as our views concerning the necessity of tyrosine for the biosynthesis of adrenaline. Nevertheless this is not the only important *rôle* which has been assigned to cysteine and its derivatives. The high sulphur content of epithelial structures has frequently attracted the attention of biochemists. Thus hair and wool contain some 5 to 10 per cent. of cystine in combination in the protein keratin. Ward has recently shown that of numerous amino acids examined cystine is the only one showing any marked power to absorb radiations in the region of the solar ultra-violet. Its behaviour in this respect differs from that of aliphatic amino acids, and its general absorption is as strong as that of phenylalanine. It is suggested that it may therefore possess a cyclic structure, and that its special function in epithelial tissues is to protect underlying structures from the lethal effects of an excess of the ultra-violet radiations. It is noteworthy that ultra-violet irradiation is accompanied by the development of pigmentation (sunburn), and that the pigments so formed are probably sulphur-containing melanins. It would be of interest to know what effect ultra-violet irradiation had on the quantitative and qualitative aspects of cysteine metabolism in epithelial tissues. It may be mentioned that the

nitroprusside reaction is given by the cellular structures of the skin, for example, the epidermis, the hair follicles, the lining of sebaceous and sweat glands, and is stronger in the roots of healthy pigmented hairs than in those of healthy white hairs. In collagen and elastin fibres, hair shafts, and stratum corneum the reaction is negative (Kaye).

The Metabolic Transformations of Sulphur Compounds in the Organism

The action of the proteolytic enzymes on proteins containing sulphur is conveniently followed by means of the nitroprusside reaction. Egg white and frog muscle proteins, which initially give a negative reaction to this test, soon develop a positive reaction when subjected to the action of pepsin at appropriate hydrogen ion concentrations, indicating a gradual liberation of sulphydryl groups from some combination with other groups. It may be that cystine only is present in the native proteins, and no convincing evidence is yet available to indicate which of the two acids is present. In any case cystine is readily transformed into cysteine by such reactions as are involved in the heat and alcohol coagulation of proteins, and in the formation of acid or alkali metaproteins. The observation that phenylcarbamido-cystine, administered orally or subcutaneously, is excreted as phenylcarbamido-cysteine, supports the view that the first stage in the catabolism of cystine is conversion to cysteine. The two amino acids would therefore appear to be metabolically equivalent. Another suggestion has been advanced by Harris to account for the gradual liberation of sulphydryl groups during protein hydrolysis. He suggests that the SH groups may play some part in linking together individual amino acids, the possible types of linkage being that of a thiopeptide, R—CS . NH—R, or R . COOH + HS . R $\longrightarrow$ R—CO . S—R + H_2O, instead of R . COOH + H_2N . R $\longrightarrow$ R—CO . NH—R, or, again, an internal ring forma-

tion between β-sulphydryl groups and carboxyl groups, thus :

$$\begin{array}{l} CH_2 \cdot CHNH_2 \cdot CO \\ | \\ S \end{array}$$

The action of trypsin on proteins containing sulphur, on the other hand, is not accompanied by the liberation of sulphydryl groups, as shown by the negative nitroprusside reaction given throughout. This may be due, however, to the ready conversion of cysteine to cystine in the alkaline media necessary for the action of trypsin.

An interesting by-path in sulphur metabolism is revealed by the occurrence in the bile acids of taurine, aminoethyl sulphonic acid, $CH_2NH_2 \cdot CH_2SO_3H$, in conjugation with cholic acid. There seems to be little doubt as to the origin of taurine from cysteine by a process of decarboxylation and oxidation of the sulphydryl group, but the mechanism of this transformation is not yet clear. Taurine itself appears to be resistant to further oxidation, and if administered to man or to the dog it is excreted as neutral unoxidised sulphur (see p. 232). There appears to be a taurine reserve in the animal body, since the amount of taurocholic acid secreted in the bile is not increased by administering taurine, whereas it is increased by the administration of cholic acid (Foster, Hooper, and Whipple). The output of taurocholic acid in the bile is also, as one might expect, increased by the feeding of cysteine and sodium cholate. It is possible that the oxidation of the former to taurine may take place after the combination with cholic acid, although the demonstrable occurrence of free taurine in certain tissues and the taurine reserve already mentioned would seem to render such a hypothesis at least unnecessary. The bulk of the evidence would appear to be in favour of the view that taurine does not represent one of the normal intermediates on the path of cysteine metabolism.

The Excretion of Sulphur

In as far as the excretion of nitrogen and of sulphur compounds in the urine represents the protein catabolism of the body, one expects to find a quantitative parallelism between these two types of excretory end-products. This is shown by a more or less constant nitrogen to sulphur ratio (N : S), the fasting value of which is placed by various workers between 10 and 16. The ratio naturally varies according to the diet and metabolic activity, and thus a much higher N : S ratio than those quoted, indicating a conservation of sulphur, has been obtained on feeding meat to a dog after a prolonged fast, whilst lower ratios are observed during the period of fasting. Low ratios have also been reported after severe muscular exercise, supposedly due to the increase in tissue respiratory processes and the intimate relationship of these to cysteine (glutathione) metabolism. The behaviour of the N : S ratio under various conditions of dieting is well shown in the accompanying table, p. 230, taken from some recent experiments on the human subject reported from Cathcart's laboratory.

These figures illustrate the gradual diminution in the N : S ratio during a period of nitrogen-free dieting or during starvation. There would appear to be no attempt on the part of the body to conserve sulphur in preference to nitrogen, despite the important *rôle* which the former plays in tissue metabolism, but rather the reverse. Apparently the nitrogen compounds are as important, possibly more so, than the sulphur compounds during a crisis such as starvation. But the sudden rise of the ratio to 17·3 on again taking food after a fast is interesting, and indicates a much more marked retention of tissue sulphur than of nitrogen during the period of recovery. An inspection of these figures also reveals the fact that in each of the three experiments in which egg-albumin was fed in addition to the basal diet there followed an immediate drop in the N : S ratio, as one would expect,

and the excretion of excess sulphur was more rapid than that of excess nitrogen. On these grounds it would seem justifi-

TABLE XVI

Variations in N : S Ratio produced by Variations in Diet (After Wilson)

Day.	Diet.	Total N.	Total S.	N : S.
1	Basal, N free	4·788	0·3022	15·8
4	,, ,, ...	2·996	0·2418	12·3
5	Starving	2·492	0·2143	11·6
6	,, ...	4·872	0·4286	11·3
7	Basal, N free	6·944	0·4012	17·3
8	,, ,, ...	4·480	0·2638	16·9
15	,, ,, ...	3·332	0·2253	14·7
16	Basal + egg-albumin [1] . .	3·920	0·4876	8·0
17	Starving	5·628	0·5660	9·9
18	,, ...	7·112	0·4506	15·7
19	Basal, N free	7·532	0·5056	14·8
20	,, ,, ...	3·900	0·2693	16·7
21	,, ,, ...	2·996	0·2583	11·5
1	Basal, containing 6·4 gm. N .	5·460	0·3737	14·6
10	,, ,, ,, .	6·076	0·4561	13·6
11	Basal (6·4 gm. N) + egg-albumin [2]	7·252	0·7912	9·1
12	Basal (6·4 gm. N) . . .	8·344	0·7473	11·1
13	,, ,, . . .	6·076	0·4467	13·6
14	,, ,, . . .	6·272	0·4506	13·9
1	Basal, containing 11 gm. N .	11·116	0·610	18·2
9	,, ,, ,, .	11·680	0·582	20·0
10	Basal (11 gm. N) + egg-albumin [3]	13·888	1·198	11·6
11	Basal (11 gm. N) . . .	15·232	1·187	12·8
12	,, ,, . . .	11·312	0·7364	15·3
13	,, ,, . . .	11·704	0·747	15·6

[1] Containing 8·87 grams N and 0·96 gram S ; N : S = 9·2.
[2] Containing 10·206 grams N and 1·059 grams S ; N : S = 9·6.
[3] Containing 9·8 grams N and 1·204 grams S ; N : S = 11·6.
Basal diet consisted of 100 grams olive oil, 300 grams tapioca, 100 grams sugar.

able to assume that the sulphur portion of the protein is metabolised in advance of the nitrogen both in the catabolic and anabolic phases. In the second experiment, in which egg-albumin was superimposed on the basal diet, the material stored and broken down again is evidently rich in sulphur, since the N : S ratio is low for the eleventh and twelfth days, whilst on the thirteenth day, when the nitrogen output has returned to its basal figure, the N : S ratio is 13·6, a little below the average for the whole experiment. The balance may be analysed as follows :—

	Grams.
Nitrogen excreted above the basal level for the three days	3·440
Nitrogen ingested on first day . . .	10·206
Nitrogen retained is therefore . . .	6·766
Sulphur excreted above the basal level for the three days	0·6262
Sulphur ingested on first day . . .	1·059
Sulphur retained is therefore . . .	0·4332

The N : S ratio of the *stored* material is therefore 6·766/0·4332 = 15·2. This is very near the N : S ratio of muscle protein, and it would seem that the body has selectively retained a material which corresponds in its nitrogen and sulphur content to that of the muscle. A similar analysis of the third experiment, in which egg-albumin was superimposed on a basal diet much richer in nitrogen, gives the following figures : nitrogen stored, 2·8 grams ; sulphur lost (since there is a negative balance in this case), 0·3131 gram. Thus there was a considerable loss in sulphur and but a slight retention of nitrogen. At the high level of protein intake it seems that the body's power to store egg-albumin, both as regards the nitrogen and the sulphur, is much below that shown in the previous experiment on a lower basal nitrogen intake.

The total sulphur excreted, which we have been considering in the above experiments, is usually divided into three fractions : (*a*) inorganic sulphates, (*b*) organic or " ethereal "

sulphates, (*c*) unoxidised or " neutral " sulphur. Fraction (*a*) represents practically wholly exogenous sulphur metabolism, just as urea does that of nitrogen, and it constitutes on the average some 85 to 90 per cent. of the total sulphur excreted. In general the fluctuations of this fraction follow closely those of urea. Fraction (*b*), the organic sulphates, consists of bodies of the type of phenol and indoxyl conjugated with sodium or potassium sulphate, and their origin is ascribed in general to the detoxication of cyclic hydroxyl compounds arising from the bacterial degradation of proteins or amino acids in the intestine. The experimental method which most markedly increases the excretion of organic sulphates is the joint oral administration of cysteine and phenol. It is stated by Rhode, and confirmed by others, that the substitution of sodium sulphate or thiosulphate for the cysteine leads, in the rabbit, to no increased excretion of organic sulphates, in addition to that produced by phenol alone, but that the sulphate is quantitatively excreted as such. This might suggest that conjugation of the sulphur compound with the phenol preceded the oxidation of the former, but on the other hand, the administration of sodium sulphite did produce an increased excretion of phenol-sulphate. More recently Hele has shown that sodium sulphate, sodium hydrogen sulphite, and cysteine, administered orally together with guaiacol carbonate to the dog, are utilised with equal readiness in the synthesis of organic sulphate. There is, however, the possibility that guaiacol is a substance too far removed from the normal metabolic paths to warrant comparison with phenol in this respect, but in any case the discrepancy is interesting. Neuberg's discovery in taka-diastase, and later in the kidney and in other organs of man, of an enzyme which he has called *sulphatase*, and which hydrolyses organic sulphates, suggests a possible widening of our views on the biological *rôle* of organic sulphates. It may be that the attachment of the sulphuric acid group to certain compounds represents a definite stage in their

metabolism. As an analogy one can point to the part played by the phosphoric acid residue in relation to the utilisation of carbohydrates, and possibly also of the fats. In any event the suggestions made from time to time by Sherwin and his associates concerning the possible endogenous origin of conjugated organic sulphates are worthy of note in this connection.

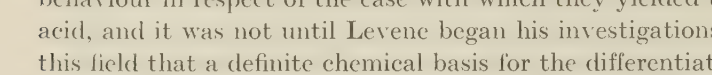
The sulphur fraction (*c*) of the urine, consisting of unoxidised sulphur, is generally assumed to be of endogenous origin, but convincing evidence on this point and concerning the question of its exact composition is lacking. Cystine or peptides containing cystine have been stated to be present, but recent quantitative determinations show that cystine is but a small component of this fraction in normal urine. In the puzzling condition of cystinuria, however, we encounter a continued excretion in the urine of considerable quantities of cystine. A small portion of this cystine seems to be of endogenous origin, whilst the major portion varies with the protein intake (Looney, Berglund, and Graves). Nevertheless cases of this pathological condition are on record in which ingested cystine was readily oxidised; included in these cases is one in which 4·6 grams of cystine isolated from the patient's own urine was completely oxidised when it was fed to him (Thiele). In other cases there may be a complete refusal to oxidise exogenous cystine, and this condition is sometimes associated with an excretion of diamines (tetra- and penta-methylene diamines), which is indicative of some impairment of protein metabolism other than that affecting the sulphur-containing amino acids. In general, however, the latter only exhibit a faulty catabolism, which must be of a highly specialised nature, since other types of sulphur compounds are oxidised and considerable quantities of inorganic sulphates appear in the urine.

Organic Sulphates Present in Tissues

Organically combined sulphuric acid is known to occur in the tissues in two forms: (1) in association with a carbo-

hydrate or derivative of a carbohydrate in the mucins and similar proteins; (2) in association with phosphatides or similar compounds not necessarily containing phosphorus. The existence of these compounds, imperfectly characterised as we must admit them to be, yet serves to show that sulphuric acid may be found in organic combinations apparently very similar in nature to those in which phosphoric acid participates.

The Conjugated Organic Sulphate Residues present in Mucoproteins.[1]—The presence of conjugated sulphuric acid in mucins was first discovered by Mörner in 1889, and he later isolated a substance which he called chondroitin sulphuric acid. Different mucins and mucoids showed a very variable behaviour in respect of the ease with which they yielded this acid, and it was not until Levene began his investigations in this field that a definite chemical basis for the differentiation emerged. It is now clear that from cartilage, tendon, aorta, and sclera mucoids a chondroitin sulphuric acid may be isolated which is constituted of two molecules of an acetylaminotalose (chondrosamine), in which one hydroxyl is replaced by the sulphate grouping, $O \cdot SO_2OH$, linked together through two molecules of glycuronic acid, thus: -

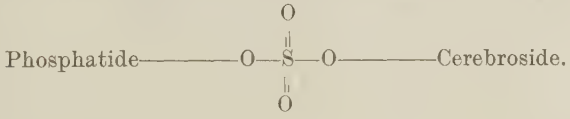


there being present a glucosidic linking between the glycuronic acid residues, both of which have their carboxyls free. The position of the sulphuric acid and amino nitrogen groups in the substituted hexosamine, and the point of attachment of the latter to the glycuronic acid, have not yet been satisfactorily determined. A very similar acid may be isolated from the vitreous humour and cornea of the eye, the mucin of the gastric mucosa, serum mucoid, ovomucoid, ovarian cysts, and Funis mucin, which, however, differs from chondroitin sulphuric acid in being much more labile, and

[1] The mucoproteins include the typical mucins and the mucoids which differ from one another in the physical character of their solutions, and of the precipitate when they are formed from an aqueous solution.

consequently more difficult to isolate without degradation. This acid, named mucoitin sulphuric acid, consistsof two molecules of acetylaminomannose (chitosamine) sulphuric acid linked together as before by two glycuronic acid residues. It differs, therefore, from the acid already described in the nature of its hexosamine, and probably also in the position of the sulphuric acid group attached to the latter. These two compounds, chondroitin and mucoitin sulphuric acids, constitute the prosthetic groups of the conjugated proteins (glucoproteins) classed as the mucoproteins, but as yet one can but speculate as to what special metabolic significance attaches to such complex amino-sugar—sulphuric acid compounds. It is possible that they might represent transformation stages between sugars and proteins, and the presence of an α-amino group in many of these sugars is suggestive in this respect, as is also the presence of the sulphuric acid group on the possible analogy, already mentioned, between phosphoric and sulphuric acids ; but at the same time the large part which such compounds play in the composition of such metabolically inert tissues as tendons and supporting tissues generally does not readily accord with this hypothesis.

The Sulphatides.—Very little is known concerning these compounds. They have already been referred to in the general classification of the lipides given on p. 132. The sulphatides comprise an obscure type of sulphur compound with properties somewhat analogous to those of the phosphatides. Their existence was first recorded by Thudichum, who reported the presence of both phosphorus and sulphur. Koch detected the presence of phosphorus, sulphur, and a reducing sugar, and suggested that the compound was a sulphate of a phosphatide and cerebroside of the type :



In 1912 Levene reported the preparation from brain of a sulphatide containing no phosphorus and having the percentage composition C = 60·9 ; H = 10·67 ; N = 2·31 ; S = 2·66 ; O = 23·46 per cent. This interesting compound does not seem to have been further investigated up to the present time.

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CHAPTER IX

THE VITAMINS

Empirical observations on the nutritional disturbances which we now ascribe to vitamin deficiency date from the latter part of the sixteenth century as is shown in the following quotation from Herbert Spencer's "The Study of Sociology."[1]

"It was in 1593 that sour juices were first recommended by Albertus; and in the same year Sir R. Hawkins cured his crew of scurvy by lemon-juice. In 1600 Commodore Lancaster, who took out the first squadron of the East India Company's ships, kept the crew of his own ship in perfect health by lemon-juice, while the crews of the three accompanying ships were so disabled that he had to send his men on board to set their sails. In 1636 this remedy was again recommended in medical works on scurvy. Admiral Wagner, commanding our fleet in the Baltic in 1726, once more showed it to be a specific. In 1757 Dr. Lind, the physician to the naval hospital at Haslar, collected and published in an elaborate work these and many other proofs of its efficacy. Nevertheless, scurvy continued to carry off thousands of our sailors. In 1780, 2,400 in the Channel Fleet were affected by it; and in 1795 the safety of the Channel Fleet was endangered by it. At length, in that year, the Admiralty ordered a regular supply of lemon-juice to the navy. Thus two centuries after the remedy was known, and forty years after a chief medical officer of the Government had given conclusive evidence of its worth, the Admiralty, forced thereto by an exacerbation of the evil, first moved in the matter. And what had been the effect of this amazing perversity of officialism? The mortality from scurvy during this long period had exceeded the mortality by battles, wrecks, and all casualities of sea-life put together!"

Spencer omits to mention the very full description of scurvy and of its treatment by lemon-juice contained in John Woodall's "Viaticum, being the Pathway to the Surgeon's Chest," (1628). Nor does he refer to Captain Cook's important contribution to

[1] Library Edition, p. 159 (1880).

the subject. On March 7th, 1776, after his return from his second voyage, Cook read to the Royal Society a detailed account of his measures to avoid scurvy. The paper is published in the Philosophical Transactions (1776, vol. lxvi, p. 402). It is interesting to note that for this contribution to science Captain Cook received the Royal Society's Copley Medal.

During the latter half of the nineteenth century a problem similar to that presented by scurvy arose in the Japanese Navy, when the disease of beri-beri, which had been promi-

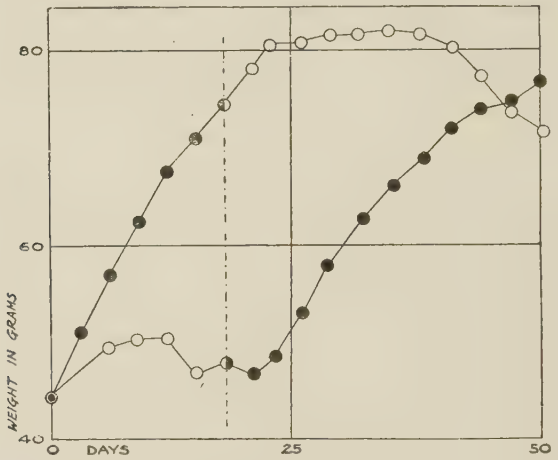


Fig. 20.—Curves showing the growth of young rats on a pure synthetic diet, and on the same diet plus a small daily allowance of milk. (After Hopkins.)

nent for some time, was ascribed to a dietary deficiency. Empirical efforts to modify the then existing diet, composed largely of polished rice, were successful in suppressing the disease when a ration of barley was substituted for a portion of the polished rice. In relation to the problem of beri-beri Eijkman, then medical officer of a prison in Java, made a series of observations, dating from 1890 to 1896, in which he noted the effect on fowls of a diet of polished rice. He found that they developed a definite pathological condition of the

nature of neuritis, and was much impressed by the resemblance of this disease to human beri-beri. He was able to show that his birds could be cured if an extract of the outer husk of the rice, which had been removed in the process of polishing, was added to the diet. Eijkman clearly excluded the possibility of the beri-beri of his birds being due to insufficiency of any of the known food components or to the presence of pathogenic organisms. In 1906 he definitely stated that the nutritional polyneuritis or beri-beri was due to the absence from the dietary of some substance present in rice polishings, and which was not a protein, fat, carbohydrate, or mineral salt. He also concluded that the presence of the unknown substance was indispensable to health. So long ago as 1881 Lunin recognised that artificial mixtures of proteins, fats, and carbohydrates, together with salts and water, cannot be used for rearing experimental animals, whereas milk, which contains these food components, is adequate for the purpose. He therefore argued that milk must contain substances, other than these five well-recognised components, which are indispensable to the growing organism. It was not, however, until 1906–7 that this important fact was definitely established by Gowland Hopkins in a series of experiments which were not published until 1912. The accompanying Fig. 20 shows some of Hopkins' results. The lower curve (up to the eighteenth day) shows the average weight in grams from day to day of eight male rats upon a pure dietary composed of purified casein, starch, lard, inorganic salts and water, whilst the upper curve shows the weight of eight similar rats taking 3 c.c. of milk per diem in addition to the pure basal diet mentioned. On the eighteenth day, marked by a vertical dotted line, the milk was transferred from one set of rats to the other. At the beginning of the experiment the rats receiving the milk showed a much greater rate of growth than the rats kept on the artificial diet, an increase altogether out of proportion to the

small amount of solid matter in the 3 c.c. of milk which they received, and which the other rats did not. The former set of rats, however, when deprived of the milk, soon ceased to grow, and in fact lost weight, whilst the under-developed rats at once began to grow rapidly when the milk was added to their deficient dietary. Just before the publication of Hopkins' results, Osborne and Mendel, and at the same time McCollum and Davis, had been working on similar dietetic problems and had arrived at conclusions very similar to those of Hopkins. The American workers showed that the milk fat, and also the residue obtained after removal of the fat, casein, and albumin from the milk, contained growth-promoting substances similar to those which Hopkins had also been able to extract from the milk by means of alcohol.

Thus we see how these early observations on the failure of normal growth of animals kept on artificial diets led to the recognition of the existence of a growth-promoting substance, whilst the still earlier work on specific diseases is responsible for the concept of two other essential substances, one having anti-neuritic, the other having anti-scorbutic functions. These substances, present in the natural foodstuffs in variable and small amounts, have been termed accessory factors, food hormones, and vitamines. The last term, suggested by Funk, was used to indicate a supposed relationship to organic nitrogen compounds which further investigation has not substantiated. At present this last term is retained, but the terminal " e " is dropped, and we now classify the three substances mentioned above as vitamins A, B, and C respectively, and differentiate them by their solubilities, A being fat soluble, and B and C water soluble. This system of terminology is preferable to a loose use of the over-worked expression " factor," and its non-committal significance is to be recommended until clearer evidence of the chemical nature of these substances is available. The system also has the advantage of being

readily expanded to accommodate the discovery of new vitamins. Thus recent work clearly establishes the occurrence in the fat-soluble group of vitamins of a growth-promoting substance, an anti-rachitic substance, and an anti-sterility substance, the two latter being now classed as vitamins D and E. Further, vitamin B has growth-promoting (pellagra-preventing) as well as anti-neuritic properties, which would now appear to be due to distinct and separate vitamins. It seems highly probable that the number of vitamins will increase with the prosecution of further researches of a more detailed nature, but at present the vitamins whose individuality has been established are as follows :—

Vitamin.	Solubility.	Effects by which vitamin is recognised.
A	Fat soluble.	Growth-promoting, essential to young animals.
B_1	Water soluble.	Anti-neuritic, essential to normal nutrition at all ages.
B_2	Water soluble.	Growth-promoting and pellagra-preventing, essential to normal nutrition at all ages.
C	Water soluble.	Anti-scorbutic.
D	Fat soluble.	Anti-rachitic, essential to young animals.
E	Fat soluble.	Anti-sterility in both sexes.

Mention must be made of another series of substances the existence of which has been postulated, and which may be related to the vitamins, more especially to vitamin B. These substances are supposed to bear a relation to the growth of simple organisms such as yeast, bacteria, and protozoa, similar to that of the vitamins to the higher animals. In the case of yeast the name " bios " has been given to this hypothetical substance. Bios has a wide distribution in vegetable extracts, and it is supposed to be characterised

by its property of stimulating the growth of yeast cells in artificial culture media. Thus it has been claimed that normal growth of yeast cells in a suitable nutrient medium will only ensue, in the absence of an outside source of bios, if the original inoculum is composed of not less than a certain minimal number of cells. The theory is that growth is catalysed by the presence of bios in sufficient amount, each cell contributing its quota ; if the number of cells in the inoculum is insufficient to make up the necessary supply of bios, growth is retarded or suppressed. Much controversy has raged round the question of the reality or otherwise and possible functions of bios in relation to the growth of yeast, and somewhat similar claims have been made concerning the dependence of the growth of bacteria, and even of green plants, on the presence of traces of preformed organic substances. Until more is known concerning the effects of small variations in the relative concentrations of known components of culture media on the development of yeast cells and bacteria, these claims must be accepted with caution. Many of the experiments involved seem to have been very inadequately controlled. In relation to the question of the existence of plant "auximones," as the substances said to stimulate plant growth have been called, Drummond and his co-workers have shown that a diatom (*Nitzschia closterium*) can grow normally in sterilised or artificial sea water, and the fresh-water alga, *Chlorella*, likewise flourishes in a solution containing inorganic salts only. Fulmer, Nelson, and White have demonstrated that yeast will grow in a synthetic artificial medium prepared from inorganic salts and pure sugar, even when the latter is a synthetic sugar. Drummond points out that it would be "an extraordinary fact if in the course of evolution, even simple green plants had lost their autotrophic nature and become to a certain extent dependent on preformed organic matter." He also draws a distinction between substances which accelerate or stimulate growth and substances which

are essential for growth. He reserves the term " vitamin " for these latter, and regards " a substance as a vitamin if it is essential to the life and well-being of an organism which does not possess the power to synthesise that substance, and also if it is organic in nature, and does not belong to any one of the three great classes of foodstuffs, proteins, fats, and carbohydrates." Bios, which is not essential for the growth of yeast, but at the most merely stimulates it, is therefore not a vitamin.

Certain of the simplest living animals, *e.g.*, the ciliate protozoön *Colpidium colpoda*, appear to resemble the plant in being independent of a source of preformed vitamins, although their growth is stimulated by substances of the nature of the hypothetical bios. As one ascends the animal scale dependence on one or more vitamins becomes more and more marked, and normal life processes become impossible in the absence of these definite substances synthesised by plant agency. As yet our information concerning the necessity of vitamins for the lower animals is very fragmentary, but the following table attempts to summarise the available data :—

Type of Animal.	Vitamins necessary.
Fly (*Drosophila*) . .	B
Tadpole and frog . . .	B A ?
Fish	A B
Bird	A B C ?
Mammal . . .	A B C D E

The animal world is dependent on the plant world for its supply of vitamins. Careful experiments have shown that the vitamins present in animal tissues and in the products of animal activity have not originated there, but have been transferred from a vegetable source. Thus the vitamins of cow's milk fat have been traced to their origin in the green foods on which the cow feeds. One of the richest sources of the fat-soluble vitamins A and D is the liver oil of the cod, and Drummond and Zilva have followed the stages in the transference of the vitamins from their vegetable source to

their final place of storage in the cod's liver. The cod is carnivorous and feeds on smaller fish, such as herrings, caplins, crustaceans, and squids, which latter in turn feed on the small organisms floating on the surface of the sea, and classed as plankton. The plankton comprises minute crustaceans, worms, decapods, copepods, and numerous larval forms, which nourish themselves at the expense of the minute chlorophyllaceous diatoms which abound in the surface layers of the sea. The vitamins of the cod's liver can be traced backwards through this chain of animals, each feeding on smaller animals, to their source in the minute plants, the diatoms. Diatoms have been grown in the laboratory, as we have seen, in sterile sea water or salt solutions, and in the presence of diffuse sunlight they are capable of forming vitamins from the purely inorganic constituents of the medium in which they grow. Fresh-water algæ and yeasts likewise flourish under these conditions, and at the same time form vitamins capable of restoring to rats kept on a vitamin-free diet their power to grow normally.

Vitamin A, the Growth-promoting Fat-soluble Vitamin

In the animal sources in which this vitamin was first found, namely in butter fat, egg-yolk fat, cod-liver oil, and ox fat, it occurs dissolved in the fat, along with which it is extracted by means of the usual fat solvents. In the plant where the vitamin is synthesised its association with fats is by no means so universal as it is in the animal, and, indeed, as it occurs in the plant it seems to be much less freely soluble in fat solvents than when it occurs in animal fat. This may be due to its association in the plant with other molecules which are not themselves fat-soluble. The absence of the vitamin from practically all vegetable oils is noteworthy in this connection. In 1914 McCollum and Davis showed that if butter is saponified and then shaken with olive oil the latter acquires growth-promoting properties, but Steenbock, Sell, and Buell first showed clearly that if

precautions are taken to prevent oxidation the vitamin is not affected by the saponification by means of boiling alcoholic potash of the fats in which it is present. The vitamin may therefore be recovered in undiminished activity and considerably concentrated in the non-saponifiable fraction, representing in most fats a little less than 1 per cent. of the original material. Use has been made of this resistance to alcoholic potash in attempts to isolate the vitamin. The technical difficulties are great, in part due to the minute amount of the vitamin originally present even in its richest sources, and in part due to its liability to undergo oxidative destruction during the prolonged manipulations involved. It has not yet been found possible to identify the vitamin as a distinct chemical substance, but Drummond and his co-workers have accumulated much interesting information concerning the chemical composition of the unsaponifiable substances with which the vitamin is associated, in the course of which work highly concentrated preparations of the vitamin have been obtained. Claims have been made by Takahashi to have isolated vitamin A in a state of purity, but on the basis of Drummond's work there is no doubt that these preparations represent substances which are themselves inactive, but with which the vitamin is closely associated. These substances, constituents of the non-saponifiable fraction of natural oils and fats, we must now consider.

The saponification of the oil or fat is achieved by means of boiling alcoholic potassium hydroxide, followed by a subsequent treatment with sodium ethoxide. These hydrolyses are carried out in an atmosphere of nitrogen to prevent oxidative loss of the vitamin. The non-saponifiable residue of the original oil contains considerable amounts of cholesterol, and when carefully prepared it is free from all trace of fatty acids, iodine, and nitrogen. It contains the whole of the vitamin present in the original oil. The cholesterol is removed from this fraction without impairing the activity

of the vitamin, by crystallisation from methyl alcohol at low temperatures (not below zero), and by precipitation of the cholesterol which still remains by means of digitonin. The residue from these treatments consists of a red-brown oil which still retains the full activity of the vitamin, all the other products separated so far being quite inactive. A further purification of the vitamin-bearing fraction may be achieved by distilling it in superheated steam in an atmosphere of nitrogen. Under these conditions the vitamin is carried over with other volatile products, and the residue of resins left in the still is found to be inactive. It is preferable, however, to subject the cholesterol-free portion to distillation in a high vacuum. In the course of their recent work on cod-liver oil, Drummond, Channon, and Coward obtained from a large-scale preparation some 750 grams of unsaponifiable matter, from which they separated 370 grams of cholesterol. They then fractionally distilled part of the residue in a high vacuum, and obtained the following series of fractions. Distillation was more or less continuous over the whole temperature range and there was no well-marked separation. The first fraction to be collected consisted of impurities which had accumulated from the various solvents which had to be used in very large quantities. This fraction is neglected.

TABLE XVII

Fractionation of the Non-Saponifiable Residue of Cod-Liver Oil

Fraction.	Temperature Range.	Quantity.	Iodine Number.	$AsCl_3$ Colour Reaction.
II. Very pale yellow oil with marked terpene-like odour.	125°–155°/3 mm.	5 grams.	114	∓
III. Pale yellow oil, terpene odour.	155°–184°/2 mm.	7 grams.	120	++
IV. Yellow oil, similar smell .	184°–190°/2 mm.	6 grams.	125	++++
V. Yellow oil, pungent terpene-like smell.	190°–220°/1–2 mm.	36 grams.	129	++++
VI. Orange-yellow oil, pungent smell.	220°–270°/1–2 mm.	27·5 grams.	149	++
VII. Reddish-brown amber-like resin.	Residue 270° —	20·5 grams.	—	—

In the last column there is recorded the intensity of the colour reaction given with arsenic chloride. This delicate colour test, which we shall discuss shortly, serves as an indication of the presence of vitamin A in the various fractions. It will be seen that the active vitamin is definitely associated with fractions IV. and V., and the further examination of these fractions is therefore a matter of great interest. There is found in these fractions, in addition to a small amount of what is possibly lipochrome pigment, the unsaturated complex hydrocarbon spinacene, with the formula $C_{30}H_{50}$, and a saturated solid alcohol with a melting point of about 60°, which is probably batyl alcohol, $C_{20}H_{42}O_3$. The bulk of the active fractions consists of an unidentified unsaturated alcohol or alcohols with a molecular weight of about 300, and an iodine number of about 103. From other fish liver oils oleyl alcohol, $C_{18}H_{36}O$, selachyl alcohol, $C_{20}H_{40}O_3$, and batyl alcohol, $C_{20}H_{42}O_3$, have been isolated in the non-saponifiable fraction, and these, together with the alcohol phytol, $C_{20}H_{40}O$, which is present in the chlorophyll molecule, prove to be quite inactive in re-establishing growth when fed to rats maintained on a deficient diet. Spinacene and the saturated solid alcohol isolated from cod-liver oil are likewise inactive, and there is no reason to believe that lipochrome pigments, when carefully purified, have any growth-promoting properties. The substance which Takahashi has claimed to be vitamin A, which he has called " Biosterin," and to which he assigns the formula $C_{27}H_{46}O_2$, probably represents mixtures of the products discussed above, in which the vitamin is present in a highly concentrated state, just as it is in Drummond's preparations. We must therefore conclude that none of the constituents present in the non-saponifiable portion of cod-liver, and so far identified, really represents the pure vitamin A. The vitamin itself is therefore probably present even in these concentrated preparations as an " impurity " in the main bulk of unsaturated alcohols and other substances. These

facts illustrate well the great difficulties attendant on isolation and the exceedingly small amount of the vitamin which is physiologically effective. Thus a rat weighing about 100 grams will eat about 3 grams of protein, 2 grams of fat, and 10 grams of carbohydrate per diem, and to supply the necessary fat-soluble vitamins A and D it is sufficient to add to this daily ration some 20 mg. of cod-liver oil. This represents about 0·2 mg. of non-saponifiable matter and about 0·02 mg. of the fractions in which the bulk of the vitamin is found after vacuum distillation. The actual amount of vitamin which is effective therefore becomes vanishingly small.

Some information concerning the possible chemical nature of vitamin A may be gained from the observation that it is not affected in any deleterious way by the substitution of the hydroxyl group of the alcohols with which it is associated by the acetyl or benzoyl groups, or by treatment with phthalic anhydride. On the other hand, the vitamin is completely destroyed by changes which affect the unsaturated linkages, for example by bromination, by hydrogenation, or by oxidation of the unsaturated compounds of the active fractions.

The presence of vitamin A in natural fats and other substances is determined by feeding the substance under investigation in various amounts to rats or other animals—rats are selected largely because of their convenient size and relatively rapid metabolic processes—which have been kept for a period on a diet deficient in vitamin A. A suitable basal diet, which contains every necessary food component with the exception of vitamin A, is composed as follows :—

Purified casein	20 parts.
Purified starch	55 ,,
Fat (lard or hardened cotton-seed oil)	15 ,,
Mixture of crystalline inorganic salts .	5 ,,
Yeast extract	5 ,,
Lemon or orange juice . . .	A few cubic centimetres.

The mixture of inorganic salts is composed as follows (McCollum and Davis) :—

NaCl	5·19 parts.
$MgSO_4$	7·98 ,,
NaH_2PO_4, H_2O	10·41 ,,
K_2HPO_4	28·62 ,,
$CaH_4(PO_4)_2$, H_2O	16·20 ,,
Ca lactate	39·00 ,,
Iron citrate	3·54 ,,
Iodine	Trace.

In preparing this diet the casein is finely powdered, and is purified by heating, with frequent stirring, for thirty-six hours at 120° on shallow trays exposed to a current of air. Any vitamin A which is normally present in crude casein is thus oxidised and destroyed. Extraction of the casein with warm alcohol followed by light petroleum is an alternative procedure. Rice starch, which is generally employed, requires no purification. As a vitamin-free fat, lard, or preferably refined hardened (*i.e.*, hydrogenated) cotton-seed oil, is used. Young rats weighing about 50 grams are kept on this diet for a period of three to four weeks, and at the end of this period they have usually ceased to grow, their weight not being in general above 90 grams. The substance to be tested for the presence of vitamin A is then added to the dietary in varying quantities until the minimum amount which will induce definite and continuous growth for a period of four weeks is ascertained. This biological method of assay is necessarily tedious, and it is therefore highly convenient that a satisfactory chemical test has been devised by Rosenheim and Drummond. This delicate colour reactions is carried out by adding 1 c.c. of pure arsenic trichloride to 1 drop of cod-liver oil and shaking the test-tube at once. The oil dissolves immediately to form a solution of a clear ultramarine blue colour, which in the course of a few seconds assumes a purple tint and gradually fades. The coloured product formed has a well-defined absorption band extending from λ 550–590, which persists for about five minutes.

This reaction is reasonably delicate, the limit being reached with 0·05 mg. of Newfoundland cod-liver oil. After saponification of the oil the cholesterol free fraction of the unsaponifiable portion gives the reaction in a dilution of 1 in 2,000,000. A complete parallelism has been found between the presence of the chromogenic substance and vitamin A so far as the investigations have been pursued, and reactions which are known to destroy the vitamin also destroy the substance which gives the colour reaction. An interesting point is that cholesterol which has acquired anti-rachitic properties through irradiation with ultra-violet light (see p. 270) does not give this colour reaction. A colour is obtained, but it differs from that given by cod-liver oil. Rosenheim and Drummond regard the new colour test as being specific for vitamin A, a view which is corroborated by other workers, and it therefore affords a further means of distinguishing the growth-promoting vitamin from the anti-rachitic vitamin. Colour reactions very similar to that given by arsenic chloride are also given by cod-liver oil in the presence of antimony trichloride, dimethyl sulphate, trichloracetic acid, acetyl chloride, and benzoyl chloride, the last two only in the presence of zinc chloride. The reaction may be utilised for the colorimetric determination of vitamin A in the following way :—

A suitable colour standard for comparison is provided by mixing 100 c.c. of crystal violet solution (1 : 10,000) with 50 c.c. of methylene blue of the same concentration, both dyestuffs being dissolved in alcohol. Twenty milligrams of Newfoundland cod-liver oil (which contains more vitamin A than the Norwegian oil), equivalent to 1 drop from a pointed glass rod 3 mm. in diameter, when dissolved in 1 c.c. of arsenic chloride, matches the standard dye solution diluted in the proportion 3 : 2. The comparison must be made within a few seconds after the addition of the reagent. After arranging a set of suitably diluted standard solutions in test-tubes, all the oils so far examined were ranged by

Rosenheim and Drummond in a series which agreed with their growth-promoting activity. For some purposes it is more convenient to employ antimony trichloride in chloroform solution as follows : 0·2 c.c. of a 20 per cent. solution in chloroform of the fat to be tested is added to 2 c.c. of a 30 per cent. solution of $SbCl_3$ in chloroform. This test, although it has certain manipulative advantages, is not so delicate as that afforded by the use of pure arsenic trichloride.

In view of the somewhat similar colour reactions given by cholesterol and many of its derivatives, and the possible relationship between sterols and lipochromes and their general association with vitamin A in plant tissues, Rosenheim and Drummond suggest that the arsenic chloride and similar reactions are concerned with a substance derived from these types of synthetic plant products.

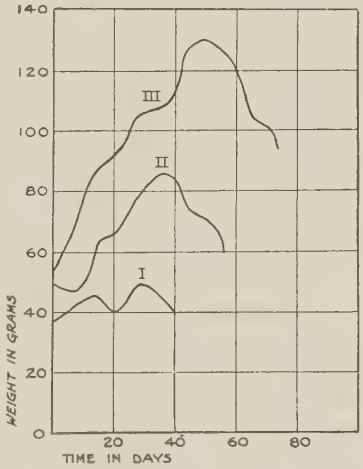


FIG. 21.—Curves showing the influence upon growth of previous feeding on diets deficient in vitamin A. (After Sherman.)

Using the above described colour test in association with biological feeding tests, Rosenheim and Webster have recently demonstrated the occurrence of vitamin A in the liver oils of all animals examined by them, fishes, birds, and mammals, and in many cases in amounts far exceeding those found in cod-liver oil. Thus the liver oils of the salmon and halibut are frequently found to be 100 times as rich in A as the cod oil. These oils cannot, however, be considered as commercial sources of the vitamin and, for the same reason, the extremely potent bird-liver oils (grouse, goose,

fulmar petrel) are likewise excluded. On the other hand, the liver oils of the sheep, calf, and ox, which are readily accessible, contain on the average some ten times as much A as does cod-liver oil, and are therefore some 200 to 1,000 times as potent as butter. Moreover, the mammalian oils are free from the characteristic odour and flavour of fish oils, a flavour due to the presence of the highly unsaturated clupanodonic acid, $C_{22}H_{34}O_2$. The incorporation of such liver fats with margarine would appear to be a simple commercial problem.

Physiological Effects of Lack of Vitamin A.—The need for vitamin A in the dietary of the adult is not so marked as it is in that of the young growing animal. Thus fully-grown rats have been maintained for several months on a diet free from vitamin A and have shown no appreciable loss of weight or other signs of ill-health. In part this effect may be due to the considerable capacity for storage of the vitamin possessed by the adult animal, a storage capacity much greater than that shown for vitamins B or C. Adult animals kept on a diet deficient in vitamin A do, however, show some impairment of nutrition in the form of an increased liability to contract bacterial infections and a diminished power of reproduction. In the young animal deprivation of the vitamin results in a cessation of growth which is eventually fatal. No satisfactory physiological basis for this failure of growth has yet been discovered. There is no marked sign of a disturbed fat metabolism. The definite disturbances in calcium and phosphorus metabolism resulting in defective ossification (rickets), and formerly ascribed to vitamin A, are now known to be due to lack of another fat-soluble vitamin, the anti-rachitic or D vitamin, which is usually associated with A. The commencement of failure of nutrition produced by absence of the vitamin from the dietary is not immediate, but begins to be apparent after the lapse of some time, which varies with the previous feeding of the animal, and presumably depends on the exhaustion of the

bodily stores of the vitamin. This is shown in Fig. 21, which represents the influence of previous feeding on the growth of rats placed on a diet lacking vitamin A. Curve I. represents the growth of a rat whose previous diet had contained little vitamin A (five-sixths whole wheat, one-sixth dried whole milk), curve II. is that of a rat whose previous diet

TABLE XVIII

Incidence of Eye Disease in Rats

	Total.	No. affected with Eye Disease.
On diets deficient in vitamin A . .	136	69
On diets deficient in vitamin B . .	225	0
On diets otherwise deficient . .	90	0
On diets experimental but presumably adequate	201	0
On mixed food (stock animals) . .	348	0
	1,000	69

had been richer in vitamin A (two-thirds whole wheat, one-third dried whole milk), whilst curve III. is that of a rat which had received a diet richer still in the vitamin (one-third whole meat, two-thirds dried whole milk). All three rats on the twenty-eighth day of their lives were placed on a diet entirely devoid of vitamin A. The rats died at the points at which the curves end. It will be seen that growth and duration of life varied with the amount of vitamin A in the previous diet, and therefore presumably with the amount of vitamin which the rats had been able to store during the preliminary dietetic *régime*.

In addition to the effects already mentioned, in rats and in other experimental animals, lack of vitamin A is frequently associated with a characteristic eye infection called xerophthalmia, which, if untreated, affects the cornea and may

result in total blindness. That this disease is specifically associated with deficiency of vitamin A is shown by the above results of Osborne and Mendel, obtained on their pedigree rats numbers 5,000 to 5,999 (see Table XVIII).

The eye disease appears to involve infection, and may be correlated with the general lowering of resistance shown by the animal kept on a deficient diet. But the specific nature of the association of the disease with lack of vitamin A shown in the above table is also shown by the fact that, if not too far advanced, the disease is usually cured without any other treatment by administration of vitamin A. Indeed Steenbock and Coward make use of the incidence of xerophthalmia as a sign of exhaustion of the animal's store of vitamin A in preference to the cessation of growth,

Vitamins, the Water-soluble Growth-promoting and Anti-neuritic Vitamins

The physiological effects of " vitamin B " are recognised in two ways : (1) its curative effect on, or preferably its preventive action in relation to, avian polyneuritis or mammalian beri-beri, and (2) its capacity to enable young rats to maintain a normal rate of growth, or to prevent or cure the symptoms of pellagra in rats or other animals. The distribution of water-soluble B as determined by growth-promoting properties is in the main coincident with that determined from its anti-neuritic action.

The water-soluble B vitamins occur in a wide variety of animal and vegetable substances, being present in variable amounts in practically all natural foodstuffs. The principal sources are plant seeds, bird's eggs, and yeast. The last-mentioned substance provides a very convenient source of the vitamins, since they are not affected by autolysis of the yeast cells (*e.g.*, in the preparation of certain commercial yeast extracts), nor by extraction or drying. A convenient method of concentrating the vitamins from yeast has been described by Osborne and Wakeman. Moist washed yeast is extracted

once with about 2 parts of boiling 0·01 per cent. acetic acid, and again with about half this amount. It is then centrifuged and evaporated to 0·4 part. This is made up to 52 per cent. by weight of alcohol, and the precipitate is separated and washed. The filtrate and washings are evaporated to about 6 per cent. of the volume of the original concentrated extract, and the alcohol concentration is brought up to 79 per cent. by weight. The precipitate is washed and twice reprecipitated by adding alcohol to its solution in a small volume of water. It is finally digested under alcohol, and on drying forms a friable mass which corresponds to 6·2 per cent. of the dry weight of the original yeast. It possesses all the potency of the original yeast. Despite numerous attempts to isolate the B vitamins in amount sufficient to permit of the determination of their chemical nature, reliable information is still lacking. Findlay suggests that the physiological basis of the action of the anti-neuritic vitamin is correlated with nuclear metabolism. Thus he finds that the chief change in the cells of the central nervous system of birds suffering from polyneuritis is a disappearance of the Nissl granules, which are composed of nucleoprotein. Peters has shown that nitrous acid does not destroy the anti-neuritic properties of yeast extract, which would seem to exclude (anti-neuritic) vitamin B from the group of primary amines. Recent attempts to obtain concentrated preparations of vitamin B carried out by Levene and his co-workers have resulted in considerable advances. Thus, starting from a concentrated preparation obtained according to Osborne and Wakeman's method, they carry out a further series of precipitations with basic lead acetate followed by barium hydroxide, and eventually obtain an ash-free substance containing 1·0 per cent. of nitrogen, potent in doses of 1·25 mg. *per diem*, having an elementary composition approximating to that of a carbohydrate, and yielding reducing substances on hydrolysis. By adsorption on silica, preparations are obtained containing 15 per cent,

of nitrogen and potent in daily doses of 0·1 mg. Kinnersley and Peters have also recently described the preparation of a yeast concentrate which cures and protects pigeons (polyneuritis) in doses of 0·084 mg. *per diem.*

In experimental work on mammals designed to investigate the distribution of vitamins B the basal diet used is that given on p. 248, with the substitution of butter fat for the lard or hardened cotton-seed oil, thus ensuring a supply of vitamin A, and the omission of the yeast extract containing B. The casein used must be very carefully purified either by exhaustive extraction with warm 95 per cent. alcohol or by repeated precipitation with dilute acetic acid. When rats

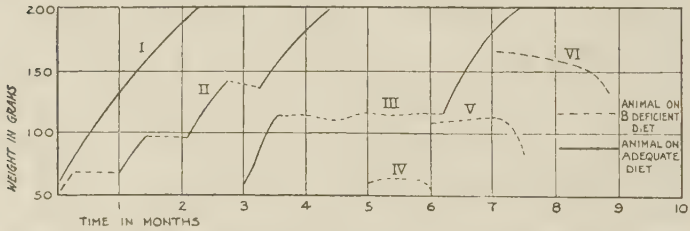


FIG. 22.—Curves showing failure of growth on a diet deficient in vitamin B, and recovery on addition of the vitamin. (After M. R. C. Report, 1924.)

are kept on such a diet the failure of growth which results differs from that produced by absence of A in occurring almost immediately, thus indicating an inability to store B, which is in marked contrast to the considerable reserve of A usually found in the body. The immediate cessation of growth is followed by a short period during which the body weight remains more or less constant; then a loss of weight commences and becomes progressively more rapid up to the death of the animal. Deprivation of B affects both young and adult animals in the same way, and although the adult may at first show a very gradual loss of weight, a rapid decline eventually appears, and soon reaches a fatal termination. We have seen, on the other hand, that

the adult is able to withstand deprivation of vitamin A with apparently but minor ill effects. The curves given in Fig. 22 illustrate the effects of withholding the water-soluble B vitamin from the dietary of rats. Curve I. is a normal control, curves II. and III. show the effects of successive periods of adequate dieting and B-deficient dieting on growth, whilst curves IV., V., and VI. show that the more mature the animal is the longer it is able to withstand the damaging effects of a deficiency of B in its diet. The animals represented in the three last curves died at the points where the curves end.

Beri-Beri.—If pigeons or other birds are kept on a diet deficient in B, severe symptoms of avian polyneuritis, which is accepted as the equivalent of human beri-beri, develop in from fifteen to twenty-five days. This is usually associated with loss of weight, but in birds the nervous symptoms predominate, and appear much sooner than they do in the mammal. The legs and wings become paralysed, and complete helplessness and death follow in from twenty-four to forty-eight hours. The administration of vitamin B in sufficient amount to birds in a completely helpless condition is usually followed by strikingly rapid cures; pigeons have been known to walk and fly within a few hours, or even less, of receiving the vitamin. A method frequently used for inducing polyneuritis in birds is to feed them on polished rice, but Simonnet has pointed out that many of the results observed under this *régime* may not be due to lack of vitamin B only, since polished rice is deficient in proteins, fat, vitamins A and C, and salts in addition to lacking B entirely. Using a diet of the following composition :—

Meat residue (extracted by boiling alcohol and ether)	11 parts.
Salt mixture	4 ,,
Cellulose	5 ,,
Agar-agar	5 ,,

Pea-nut oil (heated at 130° for three hours)	5 parts.
Butter (clarified)	10 „
Potato starch	60 „

given to pigeons in the form of pellets, Simonnet has shown that the birds do not lose weight, but avian polyneuritis develops in about thirty days, and is usually fatal in four days in the absence of treatment. It is stated that the polyneuritis developed on this deficient diet is not accompanied by paralytic or atrophic symptoms, and although there is vomiting, inability to swallow, diarrhœa, excretion of bile salts, and an increased output of uric acid, there is no marked interference with the mechanism of digestion. Simonnet considers that many of the symptoms usually included in polyneuritis are really due to inanition and multiple deficiencies. By the addition to the above diet of 0·5 gram of dried yeast *per diem* pigeons can be maintained in excellent health for many months. It would appear from the more recent work described below that Simonnet's results may be ascribed to a diet deficient in the anti-neuritic component of B only.

It has already been stated on p. 241 that what has hitherto been considered as vitamin B is a complex of at least two distinct vitamins. These are a pellagra-preventing and growth-promoting vitamin (referred to in what follows as B_2), and an anti-neuritic or beri-beri-preventing vitamin (B_1).[1] It should be stated that up till the time of writing no agreed system of nomenclature has yet been evolved, but the symbols used above will serve to differentiate the two vitamins for our present purpose. The work of Goldberger and his colleagues in America has done much to establish the correlation between pellagra, formerly ascribed to a protein deficiency, and a lack of one of the water-soluble B vitamins, and to differentiate this constituent of the vitamin complex from the anti-neuritic vitamin first discovered by Eijkman. The available evidence for

[1] Nomenclature recommended by Accessory Food Factors Committee.

ascribing the pellagra-preventing and anti-neuritic actions to distinct vitamins is as follows. Although B_1 and B_2 generally occur together in Nature, there are important quantitative differences. Thus wheat embryo is rich in B_1, but poor in B_2; many yeasts equally capable of maintaining growth (B_2) show considerable variation in anti-neuritic potency (B_1); at temperatures above 120°, B_1 is much more sensitive to inactivation than is B_2, so that by heating yeast in an autoclave at 120° for four to five hours there is obtained a preparation devoid of anti-neuritic properties, but possessing potent pellagra-preventing powers. Although both vitamins are soluble in water, B_2 is less soluble than B_1 in strong alcohol, acetone, and benzene; B_2 is more completely adsorbed, and more difficult to recover after adsorption on animal charcoal and fuller's earth than is B_1; B_1 is rapidly destroyed by alkali, whereas B_2 resists this treatment. In this country the recent work of Kon and Drummond, Hassan and Drummond, and of Chick and Roscoe corroborates and amplifies these findings. One can therefore state (1) that vitamin B_1 prevents or cures the collapse, accompanied by paralysis, which occurs in young rats in from three to four weeks, following the commencement of the period of deficient dieting; (2) that vitamin B_2 prevents the marasmus accompanied by severe skin lesions which develops in young rats after six to eight weeks of deficient dieting even when the anti-neuritic vitamin (B_1) is present to prevent the collapse which would otherwise occur earlier. It is clear in the light of this recent work that many of the earlier investigations on the attempted isolation and physiological effects of "vitamin B," based on observations of either growth-promoting or anti-neuritic properties alone, must be reconsidered in view of the dual nature of this vitamin and the differing stabilities and physical properties of its two components.

Vitamin C, the Water-soluble Anti-scorbutic Vitamin

The experimental animals which are now most generally employed in studies of vitamin C are guinea-pigs. It is an interesting fact that these animals, together with man and the apes and monkeys, are very susceptible to scurvy, whilst rats, mice, and rabbits are very much more resistant, with-

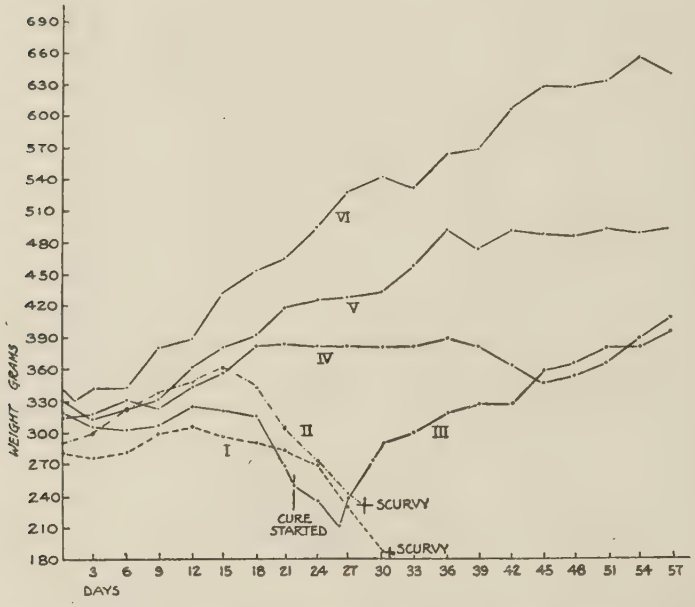


FIG. 23.—Weight charts of guinea-pigs showing the anti-scorbutic value of orange juice and fresh cabbage leaves. (After M. R. C. Report, 1924.)

standing deprivation of the vitamin for prolonged periods with relatively little sign of disturbed health. If guinea-pigs are kept on a diet of oats, bran, and a limited supply (about 40 c.c. daily) of milk autoclaved at 120° for one hour, they develop the typical effects of scurvy. The autoclaved milk provides the necessary supply of vitamin A. Normal growth takes place for a period of from fifteen to twenty days;

scorbutic symptoms then appear, loss of weight follows, and death results in about four to five weeks from the commencement of the period of deficient dieting. Typical results of experiments carried out by Chick and Hume on guinea-pigs receiving a scorbutic diet, with and without the addition of autoclaved milk, are shown in Fig. 23. Curve I. charts the weight loss on a diet of oats, bran, and water; curve II. shows a similar experiment in which the diet contained autoclaved milk; whilst curve III. shows the cure effected by adding orange juice and autoclaved milk to the diet of oats, bran, and water. In curve IV. 5 c.c. of fresh orange juice were added daily to the diet from the commencement of the experiment. Curves V. and VI. are normal growth curves obtained on diets of oats, bran, and cabbage leaves, and of oats, bran, autoclaved milk, and 3 c.c. of fresh orange juice daily. An alternative scorbutic diet proposed by Sherman and his associates is composed as follows: Oats, 59 per cent.; skimmed milk powder heated in open trays for two hours at 110° to destroy vitamin C, 30 per cent.; butter fat freshly prepared, 10 per cent.; sodium chloride, 1 per cent. This diet has the advantage of avoiding possible traces of vitamin C which might be present in autoclaved milk.

Vitamin C is found in nature in fresh fruits and vegetables in which active metabolic processes are still proceeding. Thus in resting tissues such as seeds it is usually absent, but reappears when the seed begins to germinate. The absence of vitamin C from dried seeds is in striking contrast to the occurrence of vitamin B in this source. Of fresh fruits the orange and lemon take first place as anti-scorbutic agents, whilst the juice of fresh limes is distinctly inferior. Fresh green leaves and the tomato are rich sources of the vitamin. Dried vegetables are poor in the vitamin; meat and milk are variable, but generally poor. Vitamin C is highly susceptible to destruction by oxidation, more so than vitamin A. It does not therefore survive the drying of fruits or vegetables at all well. Zilva has shown that deci-

trated lemon juice of p_H 6·8 can be boiled in an atmosphere of carbon dioxide for two hours without loss of potency, whereas similar treatment for one hour in a current of air destroys more than 75 per cent. of the vitamin. In more acid solutions of p_H 2·2 to 2·4 the loss in the presence of air is much less, but on the other hand in alkaline solutions of p_H 12 the vitamin loses 80 per cent. of its activity in thirty

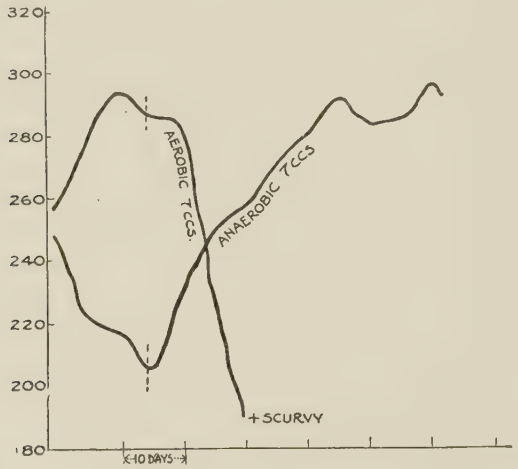
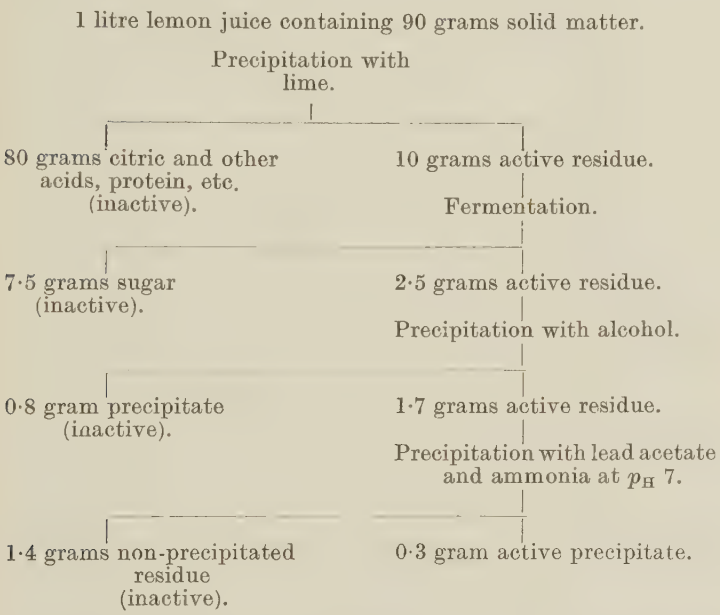


FIG. 24.—Effect of exposure to air on decitrated lemon juice kept at p_H 12. The anti-scorbutic properties of the portion exposed to air have been lost, whilst the juice kept out of contact with air is still strongly anti-scorbutic. (After Zilva.)

minutes at room temperature, and is completely destroyed at room temperature in twenty-four hours at this reaction. The practice of boiling vegetables in the presence of a little sodium carbonate or bicarbonate would therefore seem to be strongly contra-indicated. In the absence of air or oxygen no appreciable loss of vitamin C occurs at ordinary temperatures even in alkaline solutions. At higher temperatures a slow heat inactivation takes place even in the absence of oxygen. Fig. 24 gives the weight curves of two sets of

guinea-pigs, both of which were receiving a scorbutic diet. From the points marked by the dotted lines one set received daily doses of 7 c.c. of decitrated lemon juice which had been standing at room temperature for twenty-four hours at p_H 12 and freely exposed to the air. It will be seen that this preparation had no anti-scorbutic properties, since the animals rapidly lost weight and died of scurvy about the


1 litre lemon juice containing 90 grams solid matter.
Precipitation with lime.
80 grams citric and other acids, protein, etc. (inactive).
10 grams active residue.
Fermentation.
7·5 grams sugar (inactive).
2·5 grams active residue.
Precipitation with alcohol.
0·8 gram precipitate (inactive).
1·7 grams active residue.
Precipitation with lead acetate and ammonia at p_H 7.
1·4 grams non-precipitated residue (inactive).
0·3 gram active precipitate.

thirtieth day. On the other hand, in the second set of animals the administration of 7 c.c. of similar decitrated juice which had been standing at room temperature under anaerobic conditions, but at the same p_H as the aerobic sample, effected a rapid cure of scurvy, and the weight of the animals rose almost at once. It will be seen from these results that vitamin C is gradually destroyed by rise of temperature in the presence of air at a rate which increases

with diminishing hydrogen ion concentration, whilst in the absence of air the rate of inactivation is greatly decreased.

In a series of investigations which are being carried out at the Lister Institute in London by Zilva, considerable information is being gained in relation to the chemical nature of vitamin C. Concentrated preparations of the vitamin have been obtained from lemon juice by removing the citric and other acids by precipitation, and the sugar by fermentation, without perceptibly affecting the potency of the juice. Such concentrated juice contains only one-thirtieth part of the total solids of the original juice, and on concentrating the juice still further and precipitating it with alcohol the vitamin is found in the supernatant liquid, from which it may be precipitated by the addition of basic lead acetate. On removing the lead from this precipitate a very potent preparation of the vitamin is obtained. This process of concentration can be summarised as shown on p. 263.

A further concentration of the vitamin may be effected by dissolving the basic lead acetate precipitate, containing the active substance from 1 litre of lemon juice, in the minimal amount of dilute acetic acid and adding neutral lead acetate. The latter reagent does not precipitate the vitamin, but removes considerable quantities of non-active matter. The purest concentrated preparations of vitamin C obtained in this way contain very little nitrogen, some 1 to 2 mg. per cent., some of which is in the form of amide and amino nitrogen. The amide nitrogen is not related to the anti-scorbutic activity. Phosphorus is present in traces only. The active preparations reduce ammoniacal silver nitrate and potassium permanganate, but the reducing properties are lost on oxidation at a much slower rate than the anti-scorbutic properties. Swede turnip juice and cabbage leaf juice may be concentrated by a treatment similar to that outlined above, and the vitamin obtained from these sources shows physico-chemical properties similar to those of the preparations from lemon. From a study of the dialysis of

active concentrated juices Zilva concludes that the active substance approximates in molecular magnitude to the dimensions of a hexose molecule, but is distinct from the reducing and nitrogenous substances present in the active preparations. The most active fractions yet obtained contain traces of iron, phosphorus, and sulphur, and these behave on dialysis like the active principle.

Vitamin D, the Anti-rachitic Vitamin

This name was first given by Funk and Dubin to the substance already mentioned, which was supposed to be necessary for the growth of yeast, but it is now definitely used to indicate the anti-rachitic vitamin present in cod-liver oil and in other natural animal fats, and satisfactorily differentiated from the growth-promoting vitamin A, also present in these fats. Vitamin D accompanies vitamin A in the non-saponifiable residue obtained in fractionating cod-liver oil, and is found in the same final fraction of complex unsaturated alcohols as vitamin A after vacuum distillation of the active residues. It is true that the growth-promoting and anti-xerophthalmic action of natural oils and fats on the one hand, and their anti-rachitic action on the other hand, run closely parallel. Nevertheless the more detailed and more carefully controlled the investigations of these properties become, the more are differences between the two properties apparent.

Vitamin D is less liable to undergo oxidative destruction when heated in the presence of air than is vitamin A. Thus cod-liver oil loses its curative action on xerophthalmia induced by lack of fat-soluble vitamin after being subjected to the action of oxygen at 100° for periods varying from twelve to twenty-eight hours. On the other hand, the anti-rachitic action of the oil is still apparent after this treatment. Again, 0·1 gram of spinach leaves is sufficient to restore the power of growth to animals kept on a deficient diet, whereas 3·0 grams will not cure rickets. Further, the anti-rachitic

action of butter is much less than that of cod-liver oil, whereas the curative action of the former fat on xerophthalmia is somewhat greater than that of the latter. Luce has recently found that, when a cow receiving a diet deficient in fat-soluble vitamins is exposed to summer sunlight in the open air, the growth-promoting properties of the milk which she yields are not significantly affected ; on the other hand, there is a distinct increase in the anti-rachitic value of the milk. The fact that the arsenic chloride test for vitamin A specifically distinguishes it from vitamin D has already been mentioned. The final proof of the individuality of vitamin D is furnished by its production from pure ergosterol by ultra-violet irradiation (see p. 272).

In the condition known as rickets the clinical appearances vary with the animal in which the disease is developed, but the most characteristic symptom is a disturbance of calcium and phosphate metabolism which results in a deficient deposition of calcium salts, chiefly calcium phosphate, so that the bones do not become sufficiently rigid to support their normal stresses, and deformation results. The histological examination reveals an imperfect calcification of the membranous and cartilaginous matrix of the bone. The osteomalacia of adults is essentially similar to rickets in the growing animal. Experimentally the condition of rickets is readily produced in puppies and in rats. In the former animals the disease is produced by cutting down the supply of fat-soluble vitamin in a diet otherwise adequate. Growth proceeds more or less normally, a feature of rickets in the human, but ossification becomes defective. In the rat it has been supposed that the rachitic condition could not generally be induced by cutting down the fat-soluble vitamin alone ; the calcium and phosphate intake had also to be reduced or thrown out of balance before obvious rachitic conditions developed, and these were generally associated with a loss of weight. Many other animals show considerable variations in respect of their liability to develop rickets under

deficient dieting. But we have seen that similar variations in different animal species are encountered in the recognised deficiency diseases produced by lack of vitamins B and C. More recently it has been found that with strict control of the diets and attention to the age of the animal selected for the experiment it is possible to induce typical rickets in young rats. McCollum and his co-workers have recently given some attention to this question, and have evolved the following two diets, on which young rats weighing from 40 to 45 grams develop severe rickets and are ready for use after having been confined to these diets for about twenty-five days. The diets are :—

Diet 4025.	Grams.	*Diet* 4026.	Grams.
Wheat germ	5·00	Wheat germ	5·0
Salt mixture 37	5·15	Salt mixture 38	4·3
Calcium carbonate	1·50	Calcium carbonate	1·5
Gelatin	10·00	Casein (purified)	20·0
Egg albumin	10·00	Gelatin	5·0
Wheat gluten	12·00	Wheat gluten	5·0
Agar-agar	2·00	Agar-agar	2·0
Dextrin	49·35	Dextrin	52·2
Butter fat	5·00	Butter fat	5·0

In both the above diets the wheat germ may be replaced by the same amount of yeast.

The salt mixtures used in the above diets are composed as follows :—

Salt Mixture 37.	Grams.	*Salt Mixture* 38.
$CaCO_3$	1·50	Identical with salt mixture 37, except that no KH_2PO_4 is added. The casein in the diet furnishes an adequate amount of phosphorus.
KCl	1·00	
NaCl	1·00	
$NaHCO_3$	0·40	
MgO	0·20	
$FeSO_4, 7H_2O$	0·20	
KH_2PO_4	0·85	

It is now generally admitted that it is possible so to adjust diets that the deciding factor as to whether or not any

animal develops rickets becomes the presence or absence from the diet of the anti-rachitic vitamin of which cod-liver oil is a potent source. This assumes that an adequate and balanced supply of the inorganic elements of the bone is forthcoming, this requirement being parallel to the necessity for an adequate supply of the specific amino acids essential for normal growth, apart from the question of the presence or otherwise of growth-promoting vitamins. It is therefore obvious that a rachitic condition will be more speedily produced by simultaneous deprivation of both the anti-rachitic vitamin and supplies of calcium and phosphorus, more especially if the deficiency begins at an early age. In the Report of the Medical Research Council of Great Britain (1924) the points of general agreement regarding the relative importance of a deficiency of calcium, phosphate, and anti-rachitic vitamin in the causation of rickets are given as follows :—

(1) An anti-rachitic vitamin in the diet corrects improper balance in the calcium and phosphorus intake, and the greater the disproportion or defect in these elements, the more important is the *rôle* of the vitamin in the prevention of rickets.

(2) Even when the calcium and phosphorus balance is good and the supply of each adequate, the absence of anti-rachitic vitamin from the diet will result in the production of imperfectly calcified bone.

The Effect of Light in Preventing Rickets.—Much recent work in relation to rickets has centred round the curative influence of direct sunlight on the disease. These beneficial results, which have been shown to be due to the ultra-violet light radiations of about 300 $\mu\mu$ or less in wavelength, and which are cut off by ordinary glass, have given rise to an interesting development in vitamin chemistry. Hess, Unger, and Pappenheimer have shown that six young rats given a diet of white flour (95 per cent.), calcium lactate (2·97 per cent.), sodium chloride (2 per cent.), ferric citrate (0·1 per

cent.), which normally produces rickets in the laboratory, did in fact develop the disease in twenty-two to thirty-four days when kept in complete darkness. Seven similar rats, taking the same diet, were kept under the same conditions, except that for a period of fifteen to twenty minutes each day they were exposed to sunlight in the open air. These all failed to develop rickets in the same period as their fellows kept in the dark. The preventive action shown by direct sunlight was obtained equally well by subjecting the rats to the ultra-violet radiations of a quartz mercury vapour lamp or of a carbon arc lamp. The sunlight lost its curative action when it was first passed through a flint-glass window before reaching the rats. It has also been observed that rats kept on a rachitic diet containing twice the optimal amount of calcium, but deficient in phosphorus and fat-soluble vitamin D, do not develop rickets if they are exposed to sunlight in the open air. Their bones are thin, but firm and completely ossified. It is therefore apparent, as we might expect, that ultra-violet irradiation will not entirely compensate for a dietetic phosphorus deficiency, but it does exert a very favourable action on the utilisation of the supplies of phosphorus which are available. Similar beneficial results on growth are obtained when rats kept on a vitamin A deficient diet are irradiated. In course of time, however, growth ceases, presumably on exhaustion of the stores of the growth-promoting vitamin in the animal's own body, and the rats decline in weight and die, despite continued ultra-violet irradiation. Since rats kept on an adequate diet, but in absolute darkness, develop perfect bones, it is apparent that the anti-rachitic vitamin is prepotent in preventing rickets, and is capable of doing so in the entire absence of light. At the same time it is also apparent that the ultra-violet region of solar and other radiations is of great importance in rectifying partial, but not absolute, lack of the dietetic components necessary for ossification. In the light of recent work, which we shall now discuss, the

process of ultra-violet irradiation must be regarded as giving rise to vitamin D from the bodily stores of certain sterols which find their way into animal tissues from plant sources.

Anti-Rachitic Properties induced in Non-active Substances by Ultra-Violet Irradiation

A remarkable and interesting extension of the observations on the curative action of ultra-violet light on rickets, which we have just discussed, was made during 1924 by Steenbock and his co-workers, when they demonstrated that it is possible to induce anti-rachitic properties in foodstuffs which are initially quite inactive in this respect by subjecting them to irradiation with ultra-violet light. This original observation of Steenbock has been confirmed and extended by many other workers. The irradiated foodstuffs acquire not only anti-rachitic properties, but also exhibit growth-promoting activity. We shall return to a discussion of this duality of function later. It is now apparent that whilst it is possible to confer anti-rachitic properties on many natural foodstuffs and vegetable oils which are lacking or deficient in vitamin D, such as commercial casein, dried milk, meals, flour, lanoline, olive oil, bean oil, linseed oil, cotton-seed oil, lettuce, spinach, etc., no such results are obtained when the following carefully purified substances are irradiated : glycerol, fatty acids, hardened vegetable oils, fats, mineral oils, terpenes, proteins, carbohydrates, salts, water, ether, hydroquinone, phloroglucinol, paraffin hydrocarbons, etc. It is therefore apparent that it is some constituent of the natural product absent from these pure products which is responsible for the formation of the anti-rachitic vitamin under the influence of ultra-violet light.

In a vegetable oil which has acquired anti-rachitic properties the active substance is found in association with the non-saponifiable fraction, in which it may be concentrated in the manner already described as having been

utilised in attempts to isolate vitamin A. The fact has emerged that the capacity of any natural product to acquire such anti-rachitic properties is correlated with its sterol content, more particularly with cholesterol and phytosterol. Both of these sterols, on being isolated from natural oils and fats or from animal or plant tissues, and on being purified, are themselves devoid of any anti-rachitic action and contain no vitamin D. But both are readily activated on being subjected to ultra-violet irradiation. Highly purified specimens of cholesterol exhibit this property. Thus Drummond and his co-workers have subjected cholesterol to three successive treatments with boiling alcoholic potash to remove all traces of fats, and have recrystallised the cholesterol twenty-seven times from alcohol, ether, and light petroleum. It then had a melting point of 148·8° (uncorr.), and specimens have been obtained melting as high as 150°. This and other similar methods of purification in which the cholesterol was finally recrystallised as the benzoate and acetate, have all resulted in preparations which still retain the power of forming the anti-rachitic vitamin. Nevertheless, recent developments of this highly interesting aspect of vitamin chemistry have demonstrated that, rigid as these precautions to ensure purity were, they were not sufficient to achieve that end.

Early in 1927, Heilbron, Kamm, and Morton were able to show in a spectrographic study of purified cholesterol before and after irradiation with ultra-violet light, that purified cholesterol contains in small amount another compound which can be accumulated in the least soluble fraction, that this substance possesses absorption bands at 293 $\mu\mu$, 280 $\mu\mu$, and 269 $\mu\mu$, whilst cholesterol itself has only general absorption, and that these bands disappear on irradiation with a concomitant appearance of anti-rachitic potency. It was suggested that the unknown substance was closely related to the precursor of vitamin D. While the work leading to this discovery was in progress, Rosen-

heim and Webster in this country, and Windaus in Germany, working in collaboration, were examining the effects of irradiating a number of cholesterol derivatives and related compounds. Amongst these was ergosterol, $C_{27}H_{42}O$, a trebly unsaturated and labile sterol found, amongst other sources, in yeast and in the fungus ergot. It was discovered by Tanret in 1890. Irradiated ergosterol proved to be a highly potent anti-rachitic substance. In the course of this work it was shown that a specimen of recrystallised cholesterol (m.p. 148–9°), when converted into the dibromide and reconverted into cholesterol by means of the reducing action of sodium amalgam and acetic acid, although its melting point was unchanged and it retained all the usual colour reactions of cholesterol, was quite unable, after irradiation, to prevent the development of rickets in rats. Further it was established that the phytosterols stigmasterol, $C_{30}H_{48}O$, and sitosterol, $C_{27}H_{46}O$, when brominated and subsequently reduced, like cholesterol, lost their power to develop vitamin D on irradiation. In short, it was clear that the provitamin was destroyed by oxidation, reduction, and bromination, and that it possessed the properties of a sterol, since it formed an insoluble additive compound with digitonin. The sensitivity of the provitamin to oxidative processes is characteristic of only one known sterol—namely, ergosterol, already mentioned. Moreover, ergosterol shows the same, but a much more intense, ultra-violet absorption as cholesterol purified in the ordinary way by recrystallisation. Re-investigating, therefore, the anti-rachitic potency of irradiated ergosterol, Rosenheim and Webster soon demonstrated that the limiting prophylactic and curative dose, for rickets developed in rats, is of the order $\frac{1}{1000}$ to $\frac{1}{2000}$ mg. *per diem*. Dr. Katharine Coward has even been able to demonstrate in rats a definite calcifying effect of $\frac{1}{10000}$ mg. of irradiated ergosterol *per diem*. Human rickets is rapidly cured by daily doses of 2 to 4 mg. *per diem*. There is, therefore, little doubt that ergosterol is

the provitamin and that its presence in all specimens of cholesterol prepared from natural sources (gall-stones, brain, eggs, etc.), in the minute proportion of 1 part in approximately 2,000 explains the development of anti-rachitic properties in cholesterol. Irradiated ergosterol is therefore easily the most potent anti-rachitic substance known, 5 mg. being equivalent to about one litre of good cod-liver oil.

The mechanism underlying the conversion of ergosterol into vitamin D remains to be elucidated. Its acetate and benzoate can be activated to the same degree as ergosterol itself, and therefore the hydroxyl group does not appear to be concerned in the change. Rosenheim and Webster have recently shown that the maximum potency is developed after 30 minutes' irradiation (using 0·1 per cent. solutions of pure ergosterol in ether or alcohol enclosed in quartz tubes filled with nitrogen or evacuated), and that the anti-rachitic activity of the reaction products does not increase *pari passu* with the disappearance of ergosterol, but remains constant up to four hours' irradiation. It is suggested that after a short initial period the formation and destruction of the vitamin proceed at the same rate, so that the activity remains constant till the available supply of ergosterol is exhausted. The comparatively long wave ultra-violet radiations (296 $\mu\mu$ is the shortest) present in sunlight or those radiations emitted by a quartz ultra-violet lamp and transmitted by vitaglass are capable of activating ergosterol. Now human skin contains some 13 to 24 per cent. of cholesterol, and Rosenheim and Webster, in investigating several hundred grams of fat extracted from pig's skin, isolated a typical specimen of cholesterol showing in marked intensity the ultra-violet absorption characteristic of ergosterol. There is therefore no obstacle to the belief that the curative action of sunlight in human and animal rickets is due to the formation, under the influence of the solar ultra-violet rays, of vitamin D from the ergosterol present in the skin. The

direct formation of vitamin D from ergosterol is the most significant advance which has been made in the chemistry of vitamins since the existence of these substances was first demonstrated. It would seem to be highly probable that the vitamin itself must soon be isolated, and its chemical nature determined.

Irradiated cholesterol or ergosterol produces a resumption of growth in rats kept on a deficient dietary. Recent work of Steenbock in America and of Drummond in this country suggests that vitamin D may itself have the property of promoting growth in addition to its well-recognised anti-rachitic functions. Thus it has been shown that although the growth response shown by some animals kept on a deficient diet may be rapid when they are given irradiated cholesterol, in others it is irregular or entirely lacking, and is not correlated in a quantitative way with the amount of the supplement which is fed. Moreover, in animals which do give a growth response to irradiated cholesterol, a retardation followed by a cessation of growth may set in after about four weeks. A large increase in the amount of the supplement of irradiated cholesterol does not produce a resumption of growth after this second cessation ; on the other hand, normal growth is resumed on administering vitamin A. It is suggested that in such cases the first cessation is due to exhaustion of the bodily stores of vitamin D at a time when the animal has not yet expended all its stores of vitamin A, and that the final cessation of growth, when no further feeding of D produced any effect, corresponds to the complete exhaustion of A from the animal's own tissues. This seems to imply that vitamin D exerts its full growth-promoting influence only in the presence of vitamin A.

These observations make it evident that many complicated relationships exist between the numerous influences which determine growth in the young animal. The discovery that vitamin D is formed when cholesterol is irradiated has been utilised by Drummond to control more strictly what consti-

tutes a vitamin A free diet, and so to assist in differentiating more clearly the relative parts played by these two vitamins in regulating growth processes. The new basal ration used by Drummond, and believed to be adequate in all respects except in vitamin A, is composed as follows :—

Purified casein . . .	15 parts.
Pure rice starch . .	70 „
Yeast extract . . .	5 „
Salt mixture . . .	5 „
Lemon juice . . .	5 „
Irradiated cholesterol . .	2 mg. daily (administered in liquid paraffin).

It is clear that a much smaller dose of irradiated ergosterol may be substituted for the 2 mg. of irradiated cholesterol.

Control experiments show that the complete exclusion of fat does not disturb the normal metabolism of the animal. It is presumably able to synthesise all the fat which it requires from the other components of the dietary. Fat is therefore excluded entirely as an unnecessary potential source of error.

Vitamin E, the Anti-sterility Vitamin

In the course of investigations on the effects of vitamin deficiency on experimental animals it has frequently been observed that, in addition to defective growth and other symptoms which have already been discussed, the reproductive powers of the animals were impaired or even entirely lost. Thus deficiency of vitamin B_2 results in marked atrophy of the testes in male animals, but females are, in general, much less affected than the males. Rats, which, as we have seen, are not very susceptible to scurvy when kept on a diet lacking in vitamin C, nevertheless produce a larger number of litters of young when the anti-scorbutic vitamin is added to their diet. More especially has a correlation between reproductive power and the fat-soluble vitamins been established. Evans and his associates have shown that rats may be reared on a diet somewhat deficient in these vitamins,

that they may grow normally under these conditions and not suffer from xerophthalmia, and yet they show a marked diminution in their reproductive powers. In the female this is associated with a prolongation of the œstrous cycle and a failure of ovulation, as revealed by the vaginal smear test. Administration of the dry powdered leaves of young alfalfa plants or of small quantities of butter fat has cured these abnormalities. It is on the whole not surprising that a lack of vitamins which eventually leads to such profound disturbances in metabolism as we have seen to be the case should first affect the reproductive capacities of the animals when the lack is not absolute. But in more recent times disturbances of reproductive functions of a characteristic type have been traced to lack of a fat-soluble vitamin to which the letter E has been assigned. If rats are kept on the following diet :—

Skimmed milk powder . .	50·0 per cent.
Dextrin	45·3 ,,
Agar-agar	2·0 ,,
Cod-liver oil	2·0 ,,
Yeast vitamin powder . .	0·5 ,,
Ferric citrate	0·2 ,,

or on a similar diet containing fat, protein, carbohydrate, salts, and vitamins A and B, growth is normal, but after a transitory period of fertility complete sterility develops. Evans and Burr have shown that the sterility is associated with characteristic changes in both male and female rats. In the former there is destruction of the germ cells, and eventually of the entire seminiferous epithelium. In the female the ovary and the process of ovulation remain normal, but during gestation the embryo, which at first develops normally, begins to show a retardation of development about the eighth day, and between the twelfth and twentieth day the fœtus dies and is completely resorbed. The maternal placenta continues to live for some time after fœtal death has occurred. These sterility effects are prevented in both male

and female by administration of vitamin E. The distribution of the vitamin has been studied by observing its curative effects on these reproductive abnormalities. The richest source so far discovered is wheat germ oil, but the vitamin is also present in high concentration in other cereal embryos, the green leaves of plants (lettuce, alfalfa, pea, etc.), and in lower concentrations in various other vegetable oils. It is present in low concentration in animal musculature and other tissues, and is present in extremely small amount in milk fat and cod-liver oil, a fact in striking contrast to the importance of these two sources of the other fat-soluble vitamins. Animal and vegetable oils therefore seem to play an inverse *rôle* to one another in relation to the distribution of the fat-soluble A and D vitamins, and the new fat-soluble E vitamin.

In rats suffering from sterility due to lack of vitamin E a curative daily dose of wheat germ oil is 25 mg., but a single massive dose of 550 mg., representing twenty-two daily doses, is also curative even when given on the fifth or sixth day of a pregnancy which would otherwise fail through fœtal resorption.

Starting from 6 kg. of wheat germ, Evans and his co-workers have extracted the fats and oils with ether, and have saponified the extract so obtained. They have shown that the vitamin passes into the non-saponifiable fraction, and, making use of a method similar to that described under vitamin A, a concentrated preparation has been obtained by removing from the non-saponifiable residue the sterols and other impurities by crystallisation and by digitonin precipitation. There is finally obtained a sterol-free oil active in single doses of from 5 to 10 mg. This has been purified by vacuum distillation, and a fraction boiling at 200° and upwards at 0·5 mm. is found to contain the bulk of the active agent. These results are therefore very similar to those obtained on purifying and fractionating cod-liver oil. As in the case of the vitamins of cod-liver oil, it is probable that

the active fraction does not consist of the pure vitamin, but is simply a fraction of the compounds originally present in the wheat germ with which the vitamin has become associated during isolation in virtue of a similarity of physico-chemical properties. The close similarity of the results obtained on fractionating cod-liver oil and wheat germ oil in the attempted isolation of their respective vitamins is of great interest.

Vitamin E is remarkably stable to heat, light, air, and chemical reactions generally. It is not destroyed by heating at 170°, by steam distillation for several hours at 180°, nor by vacuum distillation up to 233°. When kept for twelve hours at 97° in a current of air, or treated with palladium in a current of hydrogen at 75°, no destruction of the vitamin was detected. The vitamin seems therefore to be remarkably resistant to both oxidation and reduction, a further distinction from the other fat-soluble vitamins.

Rats have a considerable capacity to store vitamin E. Thus after being put on an E-free diet fertility persists for three or four months, or for two or three gestations, its rate of disappearance being the same whether or not the animal is subjected to the drain of gestation. The vitamin does not increase fertility beyond normal limits or otherwise improve the performance of the reproductive mechanism in animals already adequately supplied. Detailed studies of the distribution and physiological effects of vitamin E will be found in the recent series of papers by Sure.

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CHAPTER X

HÆMOGLOBIN AND RELATED NATURAL PIGMENTS

The pigmented compounds of which hæmoglobin is the most familiar representative are of widespread occurrence both in the animal and in the plant world. In the former they are intimately associated, either as oxygen carriers or as oxidative catalysts, with respiratory processes, and in the latter with the photosynthetical activities of the plant cell. Both the animal and the plant compounds possess a common structural unit known as a porphyrin, which is constituted of pyrrol groups. In the naturally occurring pigment the porphyrin is associated with a metal, and there is in general another group, usually but not necessarily of a protein nature, in combination with the metallo-porphyrin. The metals which Nature has apparently found most suited for the elaboration of compounds of this class are iron and magnesium. Thus iron is found in hæmoglobin, chlorocruorin, and helicorubin, all of which are of animal origin, whilst magnesium is present in the chlorophyll of green plants. In addition to these compounds, others are known to occur in the lower animals in which copper and manganese are present, the former in hæmocyanin, the latter in pinnaglobulin. Whilst these two pigments are functionally related to the hæmoglobin series, it is not yet proved that they contain a porphyrin unit within their molecule, although this seems very probable at least in the instance of hæmocyanin. The occurrence of hæmocyanin and pinnaglobulin is confined to limited groups of invertebrates. One might therefore infer that in the animal the copper and manganese pigments proved to have but a restricted range of usefulness as respira-

tory or catalytic compounds, and that Nature in the course of her evolutionary trial and error methods soon abandoned their use in favour of the much more suitable iron compounds.

The Occurrence and Physico-Chemical Properties of Hæmoglobin

Hæmoglobin is most familiar to us as the protein-pigment complex present in mammalian red blood cells. Its function there is to act as a carrier of molecular oxygen from the lungs to the tissues where the oxidative reactions occur. But in the animal world the association of hæmoglobin and related substances with oxidative processes is much wider than this would imply. Hæmoglobin itself is by no means confined to the erythrocytes of vertebrate blood, as the following quotation from a recent paper by Anson and Mirsky will show. " Hæmoglobin is found in the bloods of all vertebrates. This is about the only generalisation that can be made about its distribution. Its occurrence is very widespread throughout the animal kingdom, but it is absolutely haphazard, not following at all the usual evolutionary classifications. Hæmoglobin has been found in the starfish (*Ophiactis virescens*), and in no related forms. It has been reported in the body fluids of the larvæ of two or three insects, although not in any other insect larvæ or in the body fluids of any adult insects. The ancient and often-repeated statement that the blow-fly *Musca* contains hæmoglobin has no basis in fact. All the pulmonate snails, with one exception, contain hæmocyanin. That one exception, *Planorbis*, contains hæmoglobin. Some worms have hæmoglobin, some chlorocruorin, some neither pigment. A hæmoglobin occurs occasionally, though on the whole very rarely, in muscle ; for instance, in the striped and heart muscle of many vertebrates, and in the pharyngeal muscles of certain gastropods. Frog muscle contains none. In the parasitic worm *Ascaris*, not only the muscles, but also all the tissues except those of the generative organs, are impregnated with hæmoglobin. Apparently a very compli-

cated substance with very peculiar properties has been evolved independently again and again in Nature."

One of the most obvious properties of hæmoglobin and of similar natural pigments is the characteristic series of absorption bands which they show in the visible region of the continuous spectrum of white light. These bands serve as a convenient means of identifying the pigments and for the investigation of their physico-chemical properties. The approximate positions of the absorption bands shown by such a pigment as hæmoglobin will be familiar to readers, since these bands are readily observed by means of the simple spectroscope or the wavelength spectrometer. In recent years the introduction of the micro-spectroscope has enabled biochemists to observe the absorption spectra of pigments present in very small quantities and permits of the examination of micro-preparations of living tissues and cells. This instrument, which can be used after the manner of the ocular of an ordinary microscope, is shown in Fig. 25. It has proved specially useful in the examination of the pigments present in the tissues of small animals such as insects.

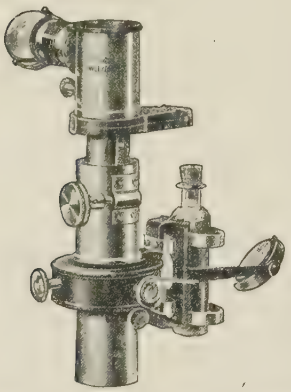


Fig. 25.—The Leitz micro-spectroscope. (Courtesy of Messrs. Ogilvy & Co.)

It is frequently essential that the precise positions of the absorption bands should be determined with accuracy, since, apart from the well-marked differences between obviously distinct pigments, in many cases the characterisation of closely related and at first sight identical pigments depends on the observation that the mean wavelengths of corresponding bands are not coincident. The determination of the mean wavelength of the absorption bands of pigments by means

of the ordinary spectroscope is inaccurate because of the breadth of the bands and the indefiniteness of their margins, and for the purpose of making this measurement with the necessary accuracy Hartridge has devised a modified instrument which he calls a reversion spectroscope. The plan of the instrument is shown in Fig. 26. In place of the ordinary

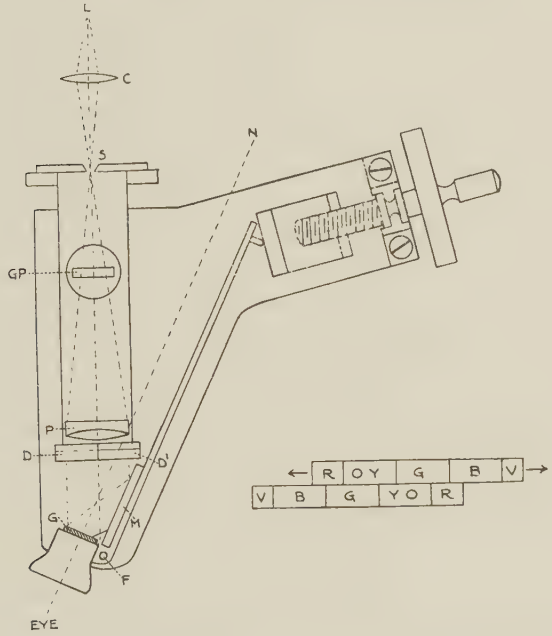


Fig. 26.—Diagram of Reversion Spectroscope. (After Hartridge.)

cross-wire, it makes use of an absorption band of the same width, mean density, and distribution of absorption as that to be measured, but reversed. The absorption band provided as an index is formed in a second spectrum reversed in direction to the first, as shown in the figure, both spectra having the same brightness and dispersion, and both being formed, the one above the other, from light that has passed

through the pigment under examination. The bands in the index spectrum are therefore similar to those in the other, no matter what may be the thickness and concentration of the pigment or the properties of the source of light. The instrument is so constructed that the index spectrum may be shifted parallel to its long axis by the manipulation of the micrometer mechanism so that the two similar bands may be moved until they appear to the observer to be in line. The micrometer index then points on the scale to the mean wavelength of the band which has been the subject of measurement. Suppose that two absorption bands be set so that the left-hand edge of one band is in coincidence with the left-hand edge of the other band above it, then the right-hand edges must also be in coincidence, since the two bands are mirror images of one another, and if λ' is the wavelength of one edge of the band and λ'' that of the other edge, then the point midway between the edges will have a wavelength of $\frac{1}{2}(\lambda' + \lambda'')$. Since when the edges of the bands are in coincidence the midway points must also be in coincidence, it is clear that the wavelength of $\frac{1}{2}(\lambda' + \lambda'')$ of one spectrum is in coincidence with that of the other, and therefore the reading given by the micrometer index will be that of $\frac{1}{2}(\lambda' + \lambda'')$. In the instrument (see Fig. 26) light from a source L is concentrated, by means of a simple condensing lens C, on to the adjustable slit of the spectroscope S, through which some of it passes to the lens P, by which it is rendered parallel. Half of the light then passes through the prism of small angle D, which deflects it slightly upwards, and reaches the surface of the grating G, by which a spectrum is formed in which the violet end is to the left and the red end to the right. The other half of the light from the collimating lens is deflected slightly downwards by the prism D′, and then reaches the surface of the steel mirror M, by which it also is reflected on to the grating, but from the opposite side of the normal N as compared with the light reaching the grating directly. In this manner a second spectrum is formed which

lies with the violet end to the right and the red end to the left, *i.e.*, in the reverse direction to the spectrum previously considered. If it were not for the interposition of the weak prisms D and D′, these two spectra would lie over one another, but the prisms are given such an angle that they lie side by side as shown in the small diagram. The spectra are shifted by rotating the grating and mirror about the fulcrum F by means of the micrometer screw shown. When a small range of wavelengths has to be covered with greater accuracy the plate-glass micrometer GP is used. This consists of a thin glass plate attached to a pointer. When the plate is set at an angle to the beam passing between the slit S and lens P, the beam is deviated to one side just as if the slit itself had been given a slight side shift, and this causes the two spectra to undergo a small side shift relative to one another. Both micrometers are calibrated against standard lines of known wavelength, so that the final reading gives directly the mean wavelength of the coincident bands.

An interesting application of this instrument has been made by Hartridge and his co-workers in determining the relative amounts of two different pigments present in a solution, *e.g.*, in mixtures of oxyhæmoglobin with carboxy- or reduced hæmoglobin. The principle upon which the application is based is as follows: If the absorption bands of two pigments have different mean wavelengths, then, if both pigments are present in the same solution and are together causing absorption of light, the measurement of the mean wavelength of the absorption bands will be found to vary with the relative concentrations of the pigments if (1) the band of one pigment is approximately similar in the form of its density curve (*i.e.*, absorption coefficients plotted against wavelength) to a band of the other pigment, and if (2) at no concentration do the bands appear separated, that is, that their mean wavelengths must not differ by more than the apparent mean width of the bands. One or both pigments may vary in concentration, and the pigments may be in the

same glass vessel or in different glass vessels through which the light passes in turn. It is well known that the two absorption bands of oxyhæmoglobin are shifted towards the violet if the oxygen be replaced by carbon monoxide, a shift which may be demonstrated with the ordinary spectroscope. Using the reversion spectroscope, the measurement of the extent of this shift has been made the basis of methods of determining quantitatively the relative amounts of oxy- and reduced hæmoglobin, or of oxy- and carboxyhæmoglobin, present in mixtures of these pigments. The method, as described by Hartridge and Roughton, is as follows: One compartment of a double wedge-shaped trough (see Fig. 27) is filled with a solution of oxyhæmoglobin, and the other with a solution of the same strength of reduced hæmoglobin. Examination in the spectroscope of the light which has passed through one end of the trough shows the bands of oxyhæmoglobin (A), whilst the other end shows the bands of reduced hæmoglobin (B), intermediate positions showing the bands of various mixtures of these substances according to their relative thickness. An additional trough is now filled with a solution of carboxyhæmoglobin and placed between the double wedge-shaped trough and the source of light so that the beam of light first passes through this single trough, and finally through the spectroscope. Suppose the thickness of the single trough multiplied by the concentration of carboxyhæmoglobin contained in it is equal to the maximum thickness of the oxyhæmoglobin compartment of the double wedge-shaped trough multiplied by the concentration of pigment in it, *i.e.*, $T[HbCO] = T_{max}[HbO_2]$. Then when the double wedge-shaped trough is shifted as far to the left as possible (A), the position of the α-band in the spectrum is the same as that of a single solution in which 50 per cent. of the hæmoglobin is combined with O_2, and the remaining 50 per cent. with CO. If the trough is shifted as far as possible in the opposite direction (B) the α-band appears more diffuse and slightly to the violet side of the correspond-

ing band of a single solution fully saturated with CO. If the trough is shifted to an intermediate position, the absorption bands also shift to a position intermediate between their two extreme positions in A and B, their exact position

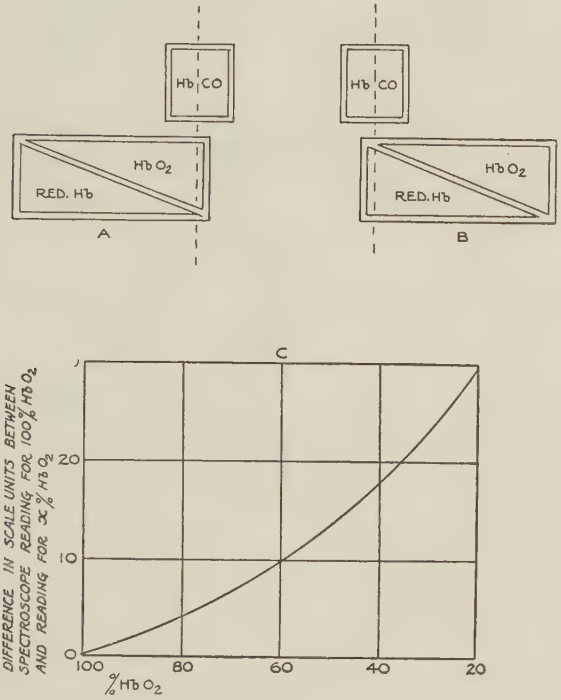


Fig. 27.—Position of troughs and calibration curve as used in Hartridge and Roughton's method of determining percentage saturation of blood pigments. (After Hartridge and Roughton.)

depending on the ratio HbO_2 : Hb traversed by the beam of light. Figs. 27 and 28 make this point clear. By leaving the HbCO trough *in situ*, and by a suitable adjustment of the double wedge-shaped trough, the position of the α-band of the spectrum can be measured for a series of different values of the ratio T[HbO_2] to T[Hb]. A calibration curve of this

kind having been obtained (see C, Fig. 27), the percentage of oxyhæmoglobin in any single solution of hæmoglobin in which there is a partial combination with O_2 can be determined. The double wedge-shaped trough is replaced by a vessel of such dimensions that when the unknown hæmoglobin is placed in this vessel the total concentration of hæmoglobin (Hb and HbO_2) in the unknown solution multi-

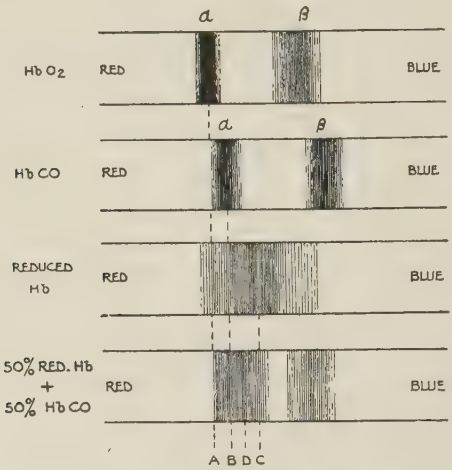
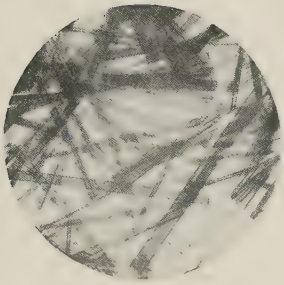


Fig. 28.—Diagrammatic representation of the relative positions of the axes (maximum absorption) of the α bands of: A, oxyhæmoglobin; B, carboxyhæmoglobin; C, reduced hæmoglobin; D, a mixture of 50 per cent. reduced hæmoglobin and 50 per cent. carboxyhæmoglobin.

plied by the thickness of the vessel is equal to $T_{max}[HbO_2]$ or [Hb] in the double wedge-shaped trough. The position of the α-band is then determined, and this with the aid of the calibration curve gives the value R of the ratio $\frac{T[HbO_2]}{T[Hb]}$ of the unknown hæmoglobin solution. But since $T_{HbO} = T_{Hb}$ = thickness of vessel containing the unknown solution then $R = \frac{[HbO_2]}{[Hb]}$ in the latter solution.



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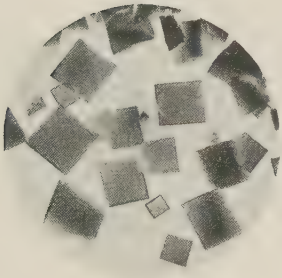

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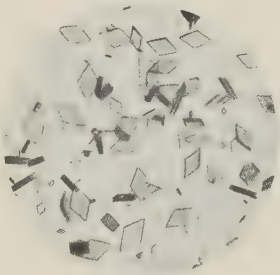

(4)

FIG. 29.—Oxyhæmoglobin crystals from :—
(1) The Sturgeon (*Acipenser sturio*), showing brachypinacoid aspect.
(2) *Necturus maculatus*, showing some twinned crystals.
(3) The Trumpeter Swan (*Olor buccinator*).
(4) The Guinea Fowl (*Numida meleagris*).
(Reproduced from Reichert and Brown.)

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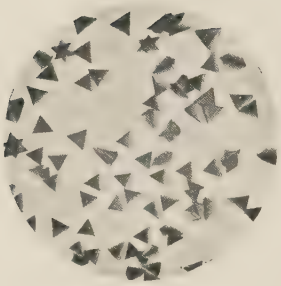

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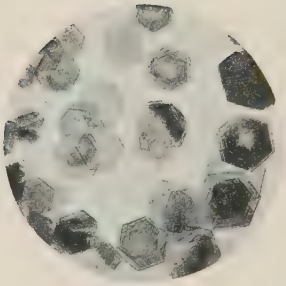

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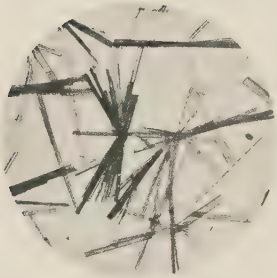

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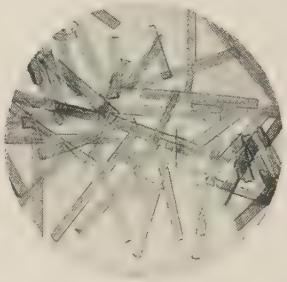

(4)

FIG. 30.—Oxyhæmoglobin crystals from :—

(1) Guinea-pig (*Cavia cutleri*), showing some twin crystals.

(2) Grey Squirrel (*Sciurus carolinensis*), showing pseudohexagonal single crystals and groups in parallel growth.

(3) Kangaroo (*Macropus giganteus*), showing lath-shaped crystals growing singly and aggregated into sheaf-like bundles.

(4) Horse (*Equus caballus*), showing long prismatic crystals.

(Reproduced from Reichert and Brown.)

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The Existence of Different Hæmoglobins in Different Animals.—The methods which we have just described have been applied to a study of the problem of the possible existence of different hæmoglobins in different species. The application has been in two main directions: (1) a study of the affinity for oxygen of the hæmoglobins from different animal sources, and (2) an exact determination of the mean wavelengths of the absorption bands of the hæmoglobins. It has been recognised for many years that the hæmoglobin from the blood of various mammals varies greatly in the ease with which it may be crystallised, and also in the form of the crystals obtained. Reichert and Brown, in their painstaking and elaborate investigations, have demonstrated this phenomenon in a very convincing manner. They have examined the crystals of oxyhæmoglobin prepared from the blood of over 100 species of mammals, and the micro-photographs reproduced in Figs. 29 and 30 illustrate some of the striking differences in crystalline form which they have recorded. These observations lend considerable support to the view that actual chemical differences exist between the various hæmoglobins.

The question of the identity or otherwise of the gas-combining power of hæmoglobins from various animals has been examined by Barcroft and Barcroft, and by Macela and Seliškar. The former investigators have shown that the hæmoglobin of *Arenicola* has an oxygen dissociation curve which shows a higher affinity for oxygen than that of human hæmoglobin, so that the gaseous exchange takes place at very low pressures. They also showed that carbon monoxide, instead of having an affinity 250 times that of oxygen, as in the case of human hæmoglobin, has only about fifty to seventy times the affinity of oxygen for the hæmoglobin of *Arenicola*. Macela and Seliškar have investigated the influence of temperature on the equilibrium between oxygen and hæmoglobin from widely different types of animals. They used dilute solutions of hæmoglobin varying in concen-

tration from 1 in 280 to 1 in 1,500, at which dilutions the dissociation curves are rectangular hyperbolæ and are not influenced by the salts present, the equilibrium between oxygen and hæmoglobin depending only on the hydrogen ion concentration and temperature. The p_H of the solutions was kept at 7·4 with a phosphate mixture. The determinations of percentage saturation were made with the reversion spectroscope by the method already described. Fig. 31

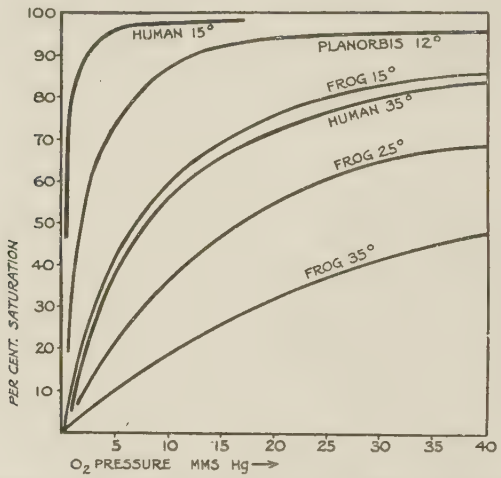


Fig. 31.—Dissociation curves of human, frog, and *Planorbis* hæmoglobins at different temperatures. (After Macela and Seliškar.)

shows some of their dissociation curves of the dilute hæmoglobin obtained from man, *Planorbis*, and the frog at various temperatures. It will be seen that the percentage saturation with oxygen at the same tensions varies greatly in these different hæmoglobins under identical physical conditions, and also in the same hæmoglobin if the temperature is altered. Not only is this the case, but it is also found that not all hæmoglobins respond in the same degree to a temperature change, that is, their temperature coefficients are not the same. Fig. 32 shows the relation

between temperature and equilibrium constant of the reaction,

$$Hb + O_2 \rightleftharpoons HbO_2,$$

for human, frog, tortoise, and *Planorbis* hæmoglobin. The logarithm of the reaction constant K is plotted against the reciprocal of the absolute temperature, *i.e.* $\frac{1}{T}$. The relative affinity of a special hæmoglobin for oxygen can be read from

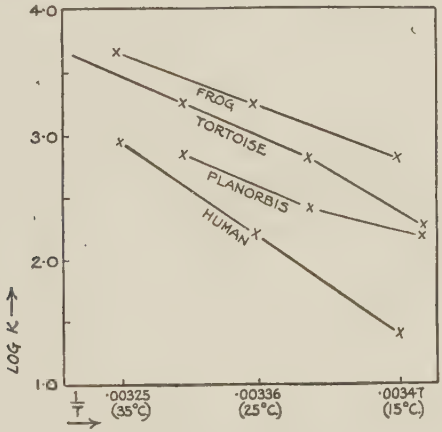


FIG. 32.—Relation between temperature and the equilibrium constant for human, tortoise, frog, and *Planorbis* hæmoglobins. (After Macela and Seliškar.)

this figure by comparing the values of log K at a given temperature, this value being greatest in forms with the lowest affinity for O_2. The value of the temperature coefficient is represented graphically by the slope of the lines. These coefficients vary greatly, the largest being the human one, which is over 5, whilst that of the frog is under 3, and that of *Planorbis* probably less. These disparities have, as Barcroft has pointed out, great biological interest. He expresses this as follows: "The temperature of the higher

mammals (apart from hibernating animals) undergoes very slight variations ; if such variations are to produce any effect of significance on the dissociation curve, clearly the curve must be very sensitive. On the other hand, the frog, in some countries, may change in temperature perhaps 30° as between the winter and the summer, in which case hypersensitiveness of its dissociation curve might be entirely out

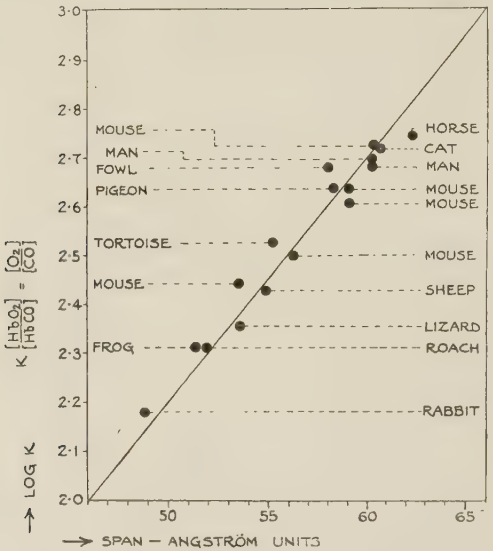


Fig. 33.—" Span " diagram of various mammalian and sub-mammalian hæmoglobins. (After Barcroft.)

of place. Clearly it is a biological necessity that the oxygen dissociation in forms which exhibit a high degree of poikilothermicity should be adapted to the general conditions of the animal, and therefore, from the biological standpoint, the factors to which the temperature coefficient of K must conform are quite different from those in homoiothermic animals."

An exact determination of the mean wavelengths of the

absorption bands of the oxy- or carboxy-derivatives of various hæmoglobins reveals the fact that slight but significant differences exist. Quantitative measurements of this phenomenon are usually made on the α-band, since it is the sharper and narrower of the two bands. It is possible to correlate these spectral differences with the varying affinity for gases shown by various hæmoglobins, and which we have discussed in the preceding paragraph. In the following table the results of Barcroft and his associates are given. It records the relative position of maximum density of the oxy- and carboxyhæmoglobin bands in the spectra of human hæmoglobin and that of several submammalian forms of life.

TABLE XIX

Animal.	Position of α-Band in Angström Units.		Span (S) in Angström Units.
	A in HbO_2	B in HbCO.	S = A − B
Man . .	5,769	5,709	60
Arenicola . .	5,751	5,697	54
Lumbricus . .	5,760	5,719	41
Planorbis . .	5,751	5,707	44
Chironomus .	5,782	5,726	56
Pigeon . .	5,767	5,709	58
Carp . .	5,767	5,715	52
Tortoise . .	5,771	5,716	55
Fowl . .	5,774	5,717	57
Lizard . .	5,767	5,714	53

If, as supposed above, the position of maximum absorption of the α-band of oxyhæmoglobin corresponds to A Angström units, and of carboxyhæmoglobin to B Angström units, then the value of A — B represents the number of Angström units through which the α-band moves when the oxygen is com-

pletely displaced by carbon monoxide according to the equation

$$HbO_2 + CO \rightleftharpoons HbCO + O_2.$$

This distance A to B is called the " span," and, as we see, it differs considerably in different vertebrate and lower animals. The value of the reaction constant K for the above equation, namely, $\frac{[HbO_2] \cdot [CO]}{[HbCO] \cdot [O_2]}$, also differs from species to species. If the length of the span in Angström units is plotted against the logarithm of this reaction constant at a given temperature, the points obtained lie more or less on a straight line, as shown in the accompanying Fig. 33. Space does not permit of a discussion of the intriguing question of the bearing of these observations on a possible chemical basis of natural selection and evolution, but it is obvious that " hæmoglobin " is not a single substance common to all the species in which it occurs. It is a complex slightly but definitely variable from species to species, the variations being adaptations of the pigment to suit the environment and needs of the individual animal.

The Chemical Basis of the Differentiation of Hæmoglobins

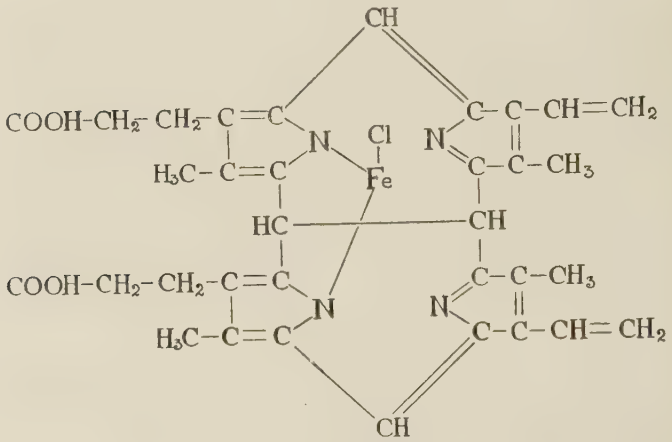
It is reasonable to suppose that the physical differences between individual hæmoglobins, which finds expression in varying crystallographic forms, absorption spectra, and dissociation curves, should be associated with distinct chemical differences. But before we can even suggest adequate chemical grounds for this differentiation we must know something more of the chemical constitution of hæmoglobin and of its related pigments.

Readers are familiar with the view that hæmoglobin is a conjugated chromoprotein in which the oxygen-carrying pigment, hæmatin, a metallo-porphyrin composed of iron in association with substituted pyrrol groups, is combined with a protein of the histone class called globin. Hæmoglobin

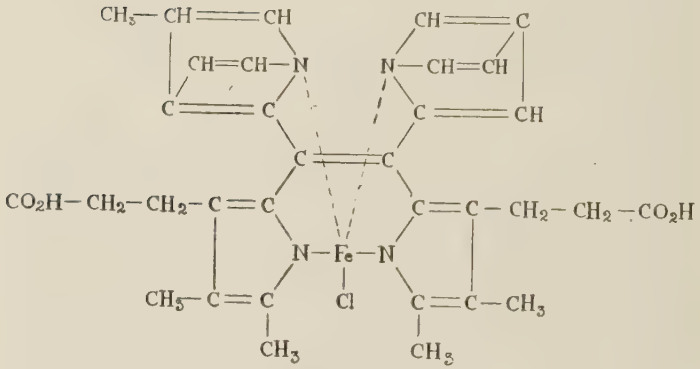
only exists as such near the neutral point, and it has been supposed until recently that the addition of dilute acid or alkali effected a disruption of the chromoprotein into its protein portion and hæmatin. By the addition of suitable reducing reagents the latter could then be converted into hæmochromogen. Further, it has been assumed that hæmatin and hæmochromogen represent respectively the oxidised and reduced forms of the iron-containing pigment group of hæmoglobin, and that these two pigments correspond to oxyhæmoglobin and reduced hæmoglobin minus their protein moieties. That these views do not give a true representation of the facts has been shown by the recent work of Barcroft and his associates. Thus Anson and Mirsky have put forward the view that hæmatin and hæmochromogen are not protein-free pigments, but that the globin of the original hæmoglobin is still present in these compounds. This suggestion is correct in the case of hæmochromogen, but, on the other hand, the more recent work of Keilin has demonstrated that alkaline hæmatin prepared from hæmoglobin and from hæmin crystals (see below), is the same substance—an iron-porphyrin compound devoid of protein. Keilin has also shown that acid hæmatin prepared from hæmoglobin is a colloidal suspension of a true hæmatin, which is not united with globin, but is protected by the latter from precipitation—hence, the earlier suggestion of Anson and Mirsky that hæmatin existed in combination with globin.

When dried blood in the presence of sodium chloride is treated with glacial acetic acid or with strong sulphuric acid in alcohol a crystalline preparation called hæmin is obtained. This substance has long been familiar to biochemists and is recognised to be the hydrochloride of the pigment portion of hæmoglobin freed from protein. The empirical formula and constitution of this substance cannot yet be regarded as settled, but the approximate composition is indicated by the suggestions of Küster ($C_{34}H_{32}O_4N_4FeCl$), of H. Fischer ($C_{34}H_{30}O_4N_4FeCl$), and of Willstätter and M. Fischer

($C_{33}H_{32}O_4N_4FeCl$). The following constitutional formulæ are those suggested by Küster and Willstätter:



Hæmin (Küster's formula).



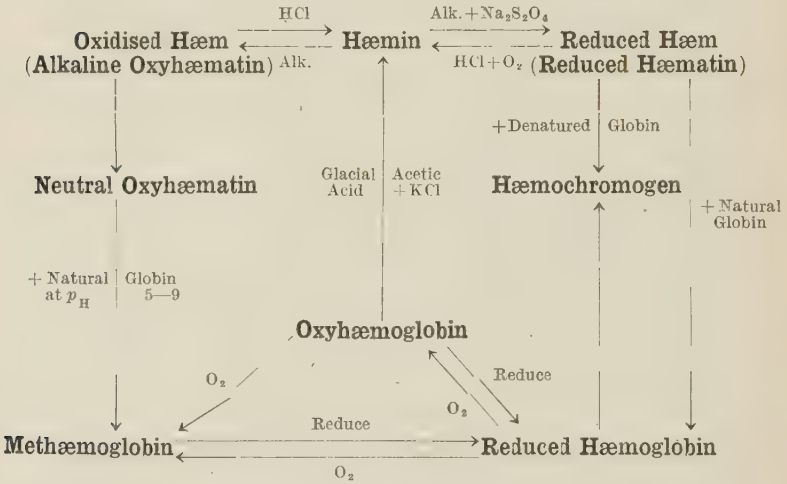
Hæmin (Willstätter's formula).

When hæmin is treated with sodium hydroxide in the presence of a reducing agent such as sodium hydrosulphite, $Na_2S_2O_4$, the corresponding base in its reduced form is

liberated from the hydrochloride. Until recently this base would have been regarded as hæmochromogen. But, as we have seen, the latter contains protein, whereas hæmin does not. Anson and Mirsky have suggested the name *hæm* for the base (Keilin's reduced hæmatin is the same substance) of which hæmin is the hydrochloride. Hæm may be obtained from hæmoglobin in the following way, which is described by the last-mentioned authors and is based on the method of Schulz. A few drops of dilute hydrochloric acid are added to a dilute aqueous solution of hæmoglobin to convert the latter into acid hæmatin. Ether and alcohol are then added in appropriate proportions and the mixture is shaken. The alcohol-water phase loses its colour. It contains globin only. The alcohol-ether phase contains the coloured component of hæmoglobin. The mechanism of this change depends upon the action of the hydrochloric acid which converts hæmoglobin into acid hæmatin—a suspension of hæm protected by globin—and this complex is readily soluble in acid. When alcohol and ether are added to the solution of acid hæmatin, the hæm passes into the alcohol-ether phase (in which it is very soluble, and in which the globin is insoluble), thereby colouring it, whilst the globin remains in the now only slightly coloured aqueous phase. The hæm prepared in this way will show the characteristic absorption spectra of hæmochromogen only after globin or a globin substitute (see p. 299) has been added to it.

On the basis of Willstätter's formula for hæmin the composition of hæm will be expressed by some such formula as $C_{33}H_{32}O_4N_4FeOH$. Hæm is insoluble in water and in acid solutions, but it dissolves in the presence of alkali. It shows a diffuse absorption spectrum in the yellow region, and with carbon monoxide it gives a spectrum of a pattern similar to that of carboxyhæmoglobin. The relationship of hæm to hæmochromogen and to reduced hæmoglobin is shown in the scheme given (p. 298). In the same scheme is shown the relationship of the oxidised form of hæm to methæmoglobin.

Methæmoglobin is the true oxide of hæmoglobin, oxyhæmoglobin being an *oxygenated*, as opposed to an *oxidised*, derivative. Hill and Holden have been able to synthesise methæmoglobin by combining neutral oxyhæmatin with natural globin at any p_H between 5 and 9. This obviously offers a means of synthesising oxyhæmoglobin from hæm and globin, since methæmoglobin may be reduced to hæmoglobin and the latter oxygenated to yield the natural respiratory pigment.



The oxygen cannot be pumped off from methæmoglobin as it can from oxyhæmoglobin, and, conversely, the formation of methæmoglobin therefore requires a higher oxidation potential than that necessary for the production of the oxygenated derivative oxyhæmoglobin. But whilst molecular oxygen is unable to convert hæmoglobin into methæmoglobin, it will convert both reduced hæm and hæmochromogen into their true oxides, oxidised hæm and hæmatin respectively. It is therefore apparent that neither hæm nor hæmochromogen is suited for the carriage of oxygen in the blood. One requires a compound which forms a loose combination

with oxygen, and from which the latter is readily liberated at the low oxygen tensions of the tissues. Nature has obtained such a substance by first combining hæm with the protein globin, so forming hæmochromogen, which on polymerisation finally yields hæmoglobin. It should be noted that a stable hæmochromogen may be obtained by combining reduced hæm with *denatured* globin, but if *natural* globin be substituted for the denatured protein reduced hæmoglobin is obtained directly, the intermediate hæmochromogen presumably polymerising at once. These relationships are shown in the scheme on p. 298. Hæmoglobin would therefore appear to be a polymer of a hæmochromogen containing natural globin, and as such is estimated to contain four hæmochromogen units with a combined molecular weight of at least 68,000. (Adair.)

Many nitrogenous substances other than globin may unite with reduced hæm to form hæmochromogens. Amongst these are other proteins, amino acids, amines, ammonia, hydrazine hydrate, pyridine, nicotine, pyrrol, etc. The hæmochromogens obtained in these instances, whilst they all give spectra very similar to the familiar hæmochromogen spectrum, differ from one another in respect of the mean wavelengths of the maxima of their α-absorption bands. Thus in ammonia-hæmochromogen (*i.e.*, the compound formed by ammonia + hæm) the position of maximum absorption of the α-band lies 25 Angström units more towards the red than the corresponding maximum of globin-hæmochromogen (*i.e.*, globin + hæm), and the other hæmochromogens show differences of a similar nature. Globin, however, has a much greater affinity for hæm than any other nitrogenous substance so far examined. Of those mentioned above only pyridine and nicotine possess an affinity at all comparable with that of globin. It will be observed that the differences between these artificial hæmochromogens are akin to those which we have seen distinguish the hæmoglobin of one species from that of another.

Many of the specific differences between individual hæmoglobins are either lost or are much less marked in the hæmochromogens prepared from them. Thus, whilst the hæmoglobins of rabbit and sheep blood have very different affinities for carbon monoxide, the corresponding hæmochromogens are not detectably different, even though the two globins present are known to be slightly different. Moreover, it would appear that slight variations in the globins, which produce measurable shifts in the absorption bands of the hæmoglobins, do not give shifts in the corresponding hæmochromogen bands which are great enough to be detected by the Hartridge reversion spectroscope. On the other hand, the corresponding hæms obtainable from the rabbit and sheep hæmoglobins mentioned above are absolutely identical, and such is also the case in all hæmoglobins so far examined. We may therefore infer that the chemical basis of the specificity of the hæmoglobins resides in the globins, and that still greater modifications in the properties of the respiratory pigment molecule would be effected by substitution of other nitrogenous substances for the globin.

The Occurrence of Compounds of Hæm in Nature

That Nature has utilised some of the possibilities mentioned in the foregoing paragraphs is shown by the occurrence of several types of pigmented compounds other than hæmoglobin, but, like it, associated with respiratory or oxidative processes. Certain of these pigments bear a close family resemblance to hæmoglobin in that they contain hæm, but, on the other hand, the globin is replaced by quite different groups. Hæmoglobin is really a highly specialised development of a particular type of pigment, and it is natural that in the lower animals and in plants, where the prevailing environmental and other conditions are very different from those of the higher animals, much more fundamental variations are encountered than those associated with the slight yet significant differences in the protein residues of the

various hæmoglobins. Amongst naturally occurring pigments which come within the scope of these observations, and which possess the same hæm as is found in hæmoglobin, are helicorubin, actiniohæmatin, and cytochrome.

Helicorubin.—Helicorubin is really a naturally occurring hæmochromogen. It is found in the liver and gut of the snail (*Helix pomatia*) and in other pulmonate molluscs. It is also found in the liver of the crayfish. Helicorubin has a spectrum of the same pattern as hæmochromogen (globin + hæm), but its bands are shifted somewhat into the red as compared with the latter pigment. A shift in the same direction is frequently met with in many of the artificial hæmochromogens which may be prepared from hæm and the various nitrogenous compounds already mentioned. On removing the compound with which the hæm of helicorubin is combined, and on replacing it with ammonia, as Anson and Mirsky have done, there is obtained an artificial hæmochromogen (ammonia + hæm) which is identical with the corresponding ammonia-hæmochromogen prepared from hæmoglobin or from any other compound in which hæm is known to occur. One must conclude that helicorubin contains the same hæm as hæmoglobin. It is noteworthy that the influence of altered p_H on the affinity of helicorubin for oxygen is just the opposite of that of a similar alteration of p_H on the affinity of hæmoglobin for oxygen. On the other hand, artificial pyridine-hæmochromogen (pyridine + hæm) shows a behaviour very similar to helicorubin in this respect. Helicorubin in a slightly acid medium, such as the gut of the snail in which it occurs, can combine loosely with oxygen, that is to say, under these conditions it forms not a stable oxide, but an oxygenated compound like oxyhæmoglobin. In contrast to its behaviour in acid solution, its affinity for oxygen in an alkaline solution is very small. Although we cannot regard the problem of the function of helicorubin in the pulmonate molluscs as definitely settled, its close physicochemical similarity to hæmoglobin certainly suggests that it

is correlated with respiration, even although it cannot be shown to circulate in a manner similar to the respiratory pigments of the higher animals.

Actiniohæmatin.—Actiniohæmatin is another pigment of the same type as helicorubin. It occurs in certain actiniæ, and from it there can be prepared an ammonia-hæmochromogen identical with that obtained from hæmoglobin and from helicorubin.

Cytochrome.—In 1884–86 MacMunn described a pigment which he called myohæmatin or histohæmatin, and which had a wide distribution in the muscles and other tissues of almost all orders of animals. He was not able to isolate this pigment in an unchanged condition, but he showed that it could exist in a reduced and in an oxidised form, the former having an absorption spectrum with four bands, whilst the latter showed no absorption. MacMunn was also able to prepare from his pigment compounds which he referred to as acid hæmatin and hæmatoporphyrin. He therefore established its analogy to hæmoglobin, but claimed that it was distinct from the latter pigment. MacMunn's results were challenged, and other workers stated that his "modified myohæmatin," from which he prepared his acid hæmatin and hæmatoporphyrin, was simply hæmochromogen derived from the hæmoglobin present in the tissues which he examined. Keilin has recently re-examined the question of the existence of a substance of the type described by MacMunn, making use of the microspectroscope and the Hartridge reversion spectroscope for the examination of the pigment as it occurs in fresh tissues. He finds that the pigment does exist and has a very much wider distribution and significance than even MacMunn anticipated. Keilin calls his pigment *cytochrome*.

In the animal kingdom cytochrome is found in all types of animals—Turbellaria, Oligochætes, Nematodes, Molluscs, Crustacea, Myriapods, Arachnids, Insecta, and vertebrates such as the frog, pigeon, guinea-pig, and rabbit. It is present

in the muscles of the last-mentioned animals together with the muscle hæmoglobin. But its distribution is not limited to the animal world, and therein lies its special significance. It is found in bacteria, in yeast, and in the higher plants. The typical appearance of the absorption spectrum of cytochrome is seen in Fig. 34, which shows the positions and approximate intensities of the bands as seen in the thoracic muscles of a bee examined in the microspectroscope. It will be observed that one of these bands really consists of three delicate bands nearly fused together. The bands vary slightly in their relative densities and in their exact positions. Cytochrome is readily converted by the oxygen of the air, or by means of potassium ferricyanide or hydrogen peroxide,

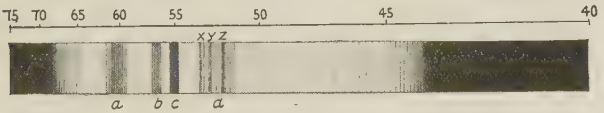


Fig. 34.—Absorption spectrum of cytochrome in thoracic muscles of a bee. (After Keilin.)

into its oxygenated form, which shows no distinct absorption. It is reduced by the tissues themselves if air be excluded, or by the addition of sodium hydrosulphite.

On adding alkali to cytochrome the appearance of the absorption spectrum is that of a typical hæmochromogen. Fischer and Schneller have obtained porphyrins from fresh yeast, autolysed yeast, and putrefying yeast, and, on treating with pyridine the residue obtained on evaporating an extract of such yeasts, a definite hæmochromogen spectrum was observed. The spectra of porphyrins have also been detected by Fischer and his co-workers in grasses and in coconut milk free from chlorophyll. It seems highly probable, as Keilin suggests, that the hæmochromogen and porphyrins which have been detected in these sources are derived from cytochrome.

Keilin has shown that cytochrome really consists of three hæmochromogens, or of two hæmochromogens and of a third similar substance. Each one of these components is responsible for one each of the *a*, *b*, and *c* bands, and also for one of the constituent bands of the composite *d* band, as shown in Fig. 35. Two of these components, namely, these which give the *b* and d_2 bands and the *c* and d_3 bands respectively, yield the same pyridine hæmochromogen (pyridine + hæm), and therefore must contain the same hæm. The exact nature of the third component has not yet been established, but it appears to contain a different hæm from the other two

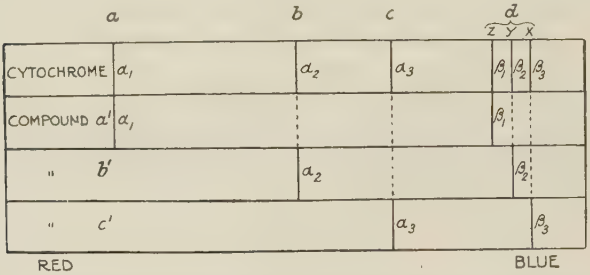


FIG. 35.—Diagram showing the three hæmochromogen compounds *a'*, *b'*, and *c''* of cytochrome. (After Keilin.)

components. We can now see that variations in the proportionate amounts of these three components, and in the extents to which each is oxygenated, will account for the differences observed in the relative intensities of the bands of cytochrome occurring in different tissues or cells. We therefore have in cytochrome a compound of hæm—the same hæm as is found in hæmoglobin—with two different nitrogen compounds, resulting in the formation of two different hæmochromogens, whilst a third component contains a substance different from, but possibly related to, hæm.

It is therefore clear that most, if not all, living animals, and also many plants, possess the constituents from which hæmoglobin may arise, and from which it would certainly

seem to have developed in the course of the evolution of the higher animals. And the widespread distribution of hæm as a constituent part of respiratory pigments both in the animal and plant worlds renders unnecessary the assumption, advanced in order to explain the haphazard distribution of hæmoglobin in Nature (*cf.* p. 281), that the power to synthesise the latter pigment has been developed again and again by independent steps in successive types of animals. The question arises as to whether this primitive form of respiratory substance really does have a functional significance similar to that of hæmoglobin in the higher animals. The readiness with which cytochrome is oxygenated by air, the equal readiness with which it is reduced by the normal activities of the cell, and the fact that, like hæmoglobin, it exhibits the properties of a thermostable peroxidase, all lend support to the view that cytochrome is associated with respiratory processes. In the muscles of the higher animals, where it occurs along with muscle hæmoglobin, it may, as Keilin suggests, represent an intermediate step in the carriage of oxygen from the lungs to the oxidative systems of the tissues, the successive " carriers " being blood hæmoglobin, muscle hæmoglobin, and cytochrome. It would therefore seem that in cytochrome there are revealed the first steps made by Nature in developing the type of respiratory pigment which has attained its highest expression in the hæmoglobin of the vertebrate animals.

Respiratory and Other Pigments which do not contain Hæm

It must not be supposed that in Nature the efforts of various groups and species of living organisms to obtain a respiratory pigment with the requisite properties for their individual needs have been confined to the type of compound which we have just discussed, namely, those resulting from the combination of hæm with a nitrogen compound. Indeed,

we have just seen that the third constituent of cytochrome is not a compound of hæm. The best-known instances of natural pigments with respiratory or similar properties, which pigments, although they do not contain hæm, are in other respects constituted on lines very similar to hæmoglobin, are furnished by the green pigments chlorocruorin and chlorophyll. The former is an animal pigment, whilst the latter is familiar as the green colouring matter of the plant world.

Chlorocruorin.—Chlorocruorin, which is red in concentrated and green in dilute solutions, is a pigment found dissolved in the plasma of certain marine worms belonging to the group of the polychætes. Munro Fox has shown that, although chlorocruorin does not contain hæm, its pigment group is composed of a porphyrin combined with iron, and must therefore be very closely allied to hæm. It is therefore not surprising to find that chlorocruorin gives rise to a series of derivatives corresponding to methæmoglobin, hæmatin, hæmochromogen, and hæmatoporphyrin. These compounds have their absorption bands shifted more into the red than those of the corresponding derivatives of hæmoglobin. Restricted as is the distribution of chlorocruorin, it nevertheless shows a striking parallelism to hæmoglobin in that specific chlorocruorins exist, with different oxygen affinities, different mean wavelengths of the positions of maximal absorption of their α-bands, and with different "spans." Thus *Myxicola*, *Spirographis*, *Sabella*, and *Branchiomma* chlorocruorins form a series with oxygen affinities diminishing in the order quoted. Since ammonia-chlorocruorochromogen has its α-band in a different position from that of ammonia-hæmochromogen, it is concluded that the porphyrin of chlorocruorin is not hæmatoporphyrin, although it is, in the natural pigment. linked to iron just as hæmatoporphyrin is in hæmoglobin.

Chlorocruorin itself exists in an oxidised and a reduced condition, and in these states shows absorption spectra analogous to those of oxy- and reduced hæmoglobin, but

with the bands shifted considerably into the red. Oxy-chlorocruorin can be reduced by exposure to a vacuum or on contact with living tissues. *Spirographis* oxy-chlorocruorin is 90 per cent. saturated at the oxygen tension of the atmosphere. The pigment is a much less efficient carrier o oxygen than is hæmoglobin, and although it is found in the blood plasma, the latter does not in the polychætes undergo a complete circulation. Nevertheless it seems highly probable that the functions of chlorocruorin are more or less closely associated with oxidative processes, and it therefore represents the parallel evolution of a substance which has a close physico-chemical similarity to hæmoglobin.

Chlorophyll.—Chlorophyll is a metallo-porphyrin derivative which has a widespread distribution in green plants, and has functions which lie at the very basis of life itself. There are present in most green leaves two chlorophylls and a number of other pigments differing fundamentally from chlorophyll and having a significance which is not yet understood. Included in these latter are carotin, which is a coloured hydrocarbon of the formula $C_{40}H_{56}$, and a number of xanthophylls having the constitution $C_{40}H_{56}O_2$. These are classed together as carotinoids or, on account of their solubility in fat solvents, as lipochromes. They do not, however, come within the scope of this chapter.

We owe most of our information concerning the chemical nature of chlorophyll to Willstätter and his school. Two chlorophylls are known, and these are distinguished by the letters *a* and *b*. Willstätter ascribes to them the following formulæ :—

$(MgN_3C_{31}H_{29})(CONH)(COOCH_3)(COOC_{20}H_{39})$	Chlorophyll *a*
$(MgN_3C_{31}H_{27}O)(CONH)(COOCH_3)(COOC_{20}H_{39})$	Chlorophyll *b*

These two components of chlorophyll exhibit, like the blood pigment, characteristic absorption spectra, which are shown in Fig. 36. It will be seen that both pigments show strong

end absorption in the violet region, an observation which has an important bearing on their functions in the green plant cell.

On acting on chlorophyll *a* with cold alkali (and chlorophyll *b* gives a similar series of results) the ester groups are

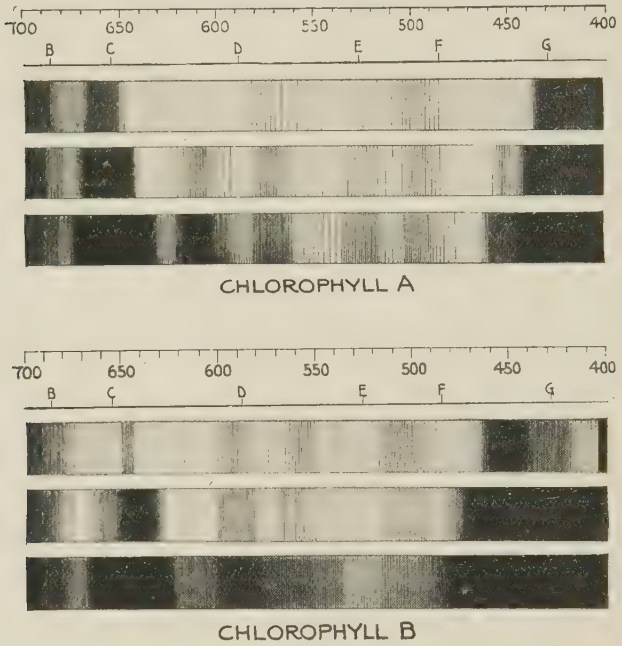
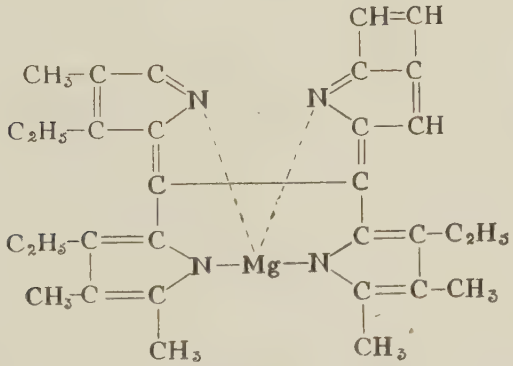


Fig. 36.—Absorption spectra of chlorophylls *a* and *b*. 0·0312 gram in ether. Thicknesses 5, 20 and 40 mm. (After Willstätter, Stoll and Utzinger.)

hydrolysed, methyl alcohol and phytol, $C_{20}H_{39}OH$, are liberated, and chlorophyllin *a* is obtained. The latter has the composition $(MgN_4C_{32}H_{30}O)(COOH)_2$, and on further treatment with hot alkali it yields various intermediate phyllins, and finally ætiophyllin, $C_{31}H_{34}N_4Mg$. Treatment with acid removes the magnesium and ætioporphyrin, $C_{31}H_{36}N_4$, is obtained. If, on the other hand, chlorophyll *a*

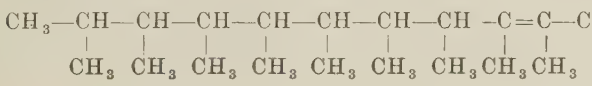
is treated with acid the magnesium is removed before the ester groups are hydrolysed, and the product obtained in this case is phæophytin *a*, $(N_4C_{32}H_{32}O)(COOCH_3)(COOC_{20}H_{39})$. Subsequent treatment with cold alkali yields methyl alcohol, phytol, and phytochlorins of the formulæ $(N_4C_{32}H_{32}O)(COOH)(COOH)$, whilst further treatment with hot alkali gives rise to a series of intermediate porphyrin derivatives, and finally ætioporphyrin identical with that obtained from chlorophyllin *a*. To ætiophyllin Willstätter and Fischer have ascribed the constitution :—



Ætiophyllin (Willstätter and Fischer).

One has only to compare the above formula with that of hæmin given on p. 296 to see the close similarity of the basal porphyrins of hæmoglobin and chlorophyll. One of the essential differences is the occurrence of iron in the former pigment, and magnesium in the latter.

Phytol, the alcohol which esterifies one of the carboxyl groups of chlorophyll, is an α-β unsaturated primary alcohol, and this interesting and curious substance is supposed to have the constitution :—



Theoretically one should obtain from a given amount of chlorophyll one-third of the weight of the latter as phytol, but in actual practice the yield is usually lower. This is probably due to the action of a hydrolytic enzyme, "chlorophyllase," which occurs in green leaves and which is active in alcoholic solution, converting the chlorophylls into chlorophyllides ; that obtained from chlorophyll *a* has the composition $(MgN_4C_{31}H_{30}O)(COOCH_3)(COOC_2H_5)$. When the chlorophyllides are treated with acids they lose magnesium and form phæophorbides of the general formula $(N_4C_{32}H_{32}O)(COOR)_2$, in which R represents the alcoholic group. Direct treatment of phæophytin *a* with hydrochloric acid would also yield a phæophorbide.

Chlorophyll and Photosynthesis.—Although the function of chlorophyll in green plants has been recognised for many years, the mechanism of the photosynthetical process in which it participates is still a matter for discussion. Its presence enables the green plant to utilise the radiant energy of sunlight, principally that of the shorter wavelengths, to synthesise complex organic compounds from carbon dioxide and water. Monosaccharides and sucrose are amongst the first recognisable products resulting from this photosynthetical activity, whilst starch appears later. According to Baeyer's original hypothesis, the first step in this process is the reduction of carbon dioxide to formaldehyde, and the subsequent steps involve the polymerisation of this substance to a hexose, and so to di-, tri-, and poly-saccharides. The reduction of carbon dioxide to formaldehyde is a markedly endothermic reaction ; hence the necessity for a large input of energy in the form of light radiations. We can therefore regard chlorophyll as a photocatalytic energy transformer (Baly and associates), or we may regard it as participating directly in the chemical transformations by which formaldehyde is formed from carbon dioxide (Willstätter and Stoll ; Maquenne). The process of photosynthesis involves the uptake of carbon dioxide and the evolution of oxygen, but at

the same time the plant cell is also respiring, that is to say, it is absorbing oxygen and giving off carbon dioxide—a process in which cytochrome may play an important part. If a correction be made for the respiratory process, the ratio of the carbon dioxide absorbed to the oxygen evolved represents that portion of the gaseous exchange of the plant which is due to photosynthesis. This is known as the "assimilatory coefficient," and its value may be obtained by gasometric methods. A series of careful measurements made by Willstätter shows that this coefficient has a value of unity under widely varying conditions of temperature, strength of illumination, content of carbon dioxide in the atmosphere, and in widely different plant species. From this important observation Willstätter and Stoll conclude that the oxygen of the absorbed carbon dioxide is entirely given off as molecular oxygen, whilst the deoxidised carbon residue remains as such, or in a hydrated form (formaldehyde), in the plant cell. Leaves which do not normally contain chlorophyll have no assimilatory power, but, on the other hand, etiolated leaves of green plants and yellow leaves show an unexpectedly marked assimilatory power. The further observations that neither the isolated chloroplasts nor chlorophyll in colloidal solution assimilate carbon dioxide all point to the presence in the leaf of some agent other than chlorophyll which regulates photosynthesis. This agent is generally supposed to be of a protein nature. Here we must leave these fascinating questions, sufficient having been said to indicate the nature of the processes in which chlorophyll participates. It will be seen that the functions of this magnesium-porphyrin compound differ considerably from those of the iron-porphyrin compounds of the hæmoglobin type.

Hæmocyanin.—We have not yet exhausted our information concerning the efforts which living organisms have made to secure substances with respiratory or similar properties. Hæmocyanin furnishes an instance of a pigment with proper-

ties analogous to those of the pigments to which we have already referred, and into whose composition a heavy metal enters. This metal is copper, and its occurrence in a compound of this type is of particular interest in view of the well-known toxic action of compounds of copper on many living organisms.

Hæmocyanin is found only in invertebrates, in which its distribution, like that of hæmoglobin, is rather erratic. It is found in the bloods of certain arthropods (crustacea and arachnids), and in certain molluscs (gasteropods and cephalopods). It can form loose combinations with oxygen, and in the form of oxyhæmocyanin it has a blue colour, whilst in the reduced condition it is colourless. It seems highly probable that here again we have a pigment which differs slightly but definitely from species to species. Thus Alsberg and Clark differentiate the hæmocyanin of *Limulus* from that of *Octopus* on the grounds of a difference in percentage composition, and in precipitation reactions with saturated magnesium sulphate, with half-saturated ammonium sulphate, and on dialysis. The two hæmocyanins also exhibit differences in their tendencies to crystallise and in their sensitivity towards acid. Redfield and Hurd also observed differences between the hæmocyanins of *Limulus* and *Loligo*. Stedman and Stedman have recently investigated the dissociation curves of hæmocyanin from the bloods of four species of crustaceæ, namely, *Homarus*, *Palinurus*, *Maia*, and *Cancer*. They have shown that, whilst the oxygen capacities of the four bloods differ widely, the shapes of the dissociation curves are very similar, and when the percentage saturation is plotted against the oxygen tension the points obtained all lie on one curve. It has also been established, from a study of the shape of the oxygen dissociation curves determined at varying p_H, that the affinity of the pigment for oxygen is at a maximum at the isoelectric point (p_H 4·7 for *Homarus* and *Cancer*), or, alternatively, that maximal affinity occurs on the acid side of the isoelectric point at a p_H corresponding

to maximal ionisation of the hæmocyanin as a protein kation. Under temperature conditions which approximate to the normal for molluscs and crustacea hæmocyanin is about 85 per cent, saturated when it is in equilibrium with oxygen tensions of 20 mm. At lower tensions dissociation would occur, and thus oxygen would be available for the tissues. It is concluded that hæmocyanin is capable of exercising real respiratory functions. According to Dhéré, the amount of copper in the blood of animals in which hæmocyanin occurs varies from 3·5 to 23·7 mg. per 100 c.c. of blood, a variation which amply accounts for the differences in oxygen capacities observed by Stedman and Stedman. Hæmocyanin has about one-fourth of the combining capacity of hæmoglobin for oxygen. The case of the snail (*Helix pomatia*) is of interest, since both hæmocyanin and helicorubin are found in its tissues.

Hæmocyanin and hæmoglobin show a very close correspondence in their percentage compositions, as is shown by the following analytical figures :—

	C.	H.	O.	N.	S.	Metal.
Oxyhæmoglobin (horse)	54·75	6·98	20·12	17·35	0·42	0·38 (Fe)
Oxyhæmocyanin (octopus)	53·66	7·33	21·67	16·09	0·86	0·38 (Cu)

This correspondence certainly suggests a close chemical similarity between the two compounds, and Philippi has, indeed, isolated an amorphous pigment group from hæmocyanin which would correspond to hæmatoporphyrin, and which gives pyrrol reactions. It is certainly an attractive hypothesis that in hæmocyanin we see Nature's experiments with a copper-porphyrin derivative, experiments which were apparently not sufficiently successful to enable hæmocyanin

to compete with the iron-porphyrins as respiratory pigments suited to the needs of the higher animals.

Pinnaglobulin.—Pinnaglobulin is a brown pigment which is present in the blood of the lamellibranch *Pinna squamosa*. It is stated to be very similar to hæmocyanin, but contains manganese in place of copper. So far as is known at present, this pigment is the result of an isolated experiment on the part of Nature, since the presence of manganese has not been reported in any other animal species. It cannot yet be stated how the manganese of pinnaglobulin is combined with the protein part of the molecule, nor is it known whether or not the metal is associated with compounds of the nature of porphyrins.

The Porphyrins

The feature which is common to the hæmoglobins, the chlorophylls, and similar substances is the presence of a porphyrin, and therefore considerable interest attaches to the occurrence of free porphyrins in Nature. We must first, however, refer to certain of the numerous porphyrins which have been prepared artificially.

The term " hæmatoporphyrin " is somewhat loosely applied to the iron-free pigments which are obtained from hæmoglobin, hæmochromogen, hæmatin, or hæmin by the action of strong acids. The exact composition of these porphyrins varies according to the method of preparation. On the basis of the formula previously assigned to hæmin, it is usual to assign to hæmatoporphyrin proper some such composition as $C_{33 \text{ or } 34}H_{36}O_6N_4$. By reduction with hydriodic and acetic acids it is converted into mesoporphyrin, $C_{34}H_{38}O_4N_4$, and with methyl alcoholic potassium hydroxide it yields hæmoporphyrin, which is either an isomer of mesoporphyrin or contains two hydrogen atoms less. The latter seems more probable, since hæmoporphyrin is converted into mesoporphyrin when it is treated with hydriodic acid in glacial

acetic acid. It would seem that neither hæmatoporphyrin nor mesoporphyrin is formed in the human body. An important artificial porphyrin is ætioporphyrin, which, as we have seen, is the product eventually obtained from chlorophyll on hydrolysing the ester groups and on removing the magnesium. But ætioporphyrin is also obtained from hæmoporphyrin when the latter is heated with soda lime, from mesoporphyrin by conversion into its phyllin followed by decarboxylation, and by the action of magnesium oxide and potassium methylate on uroporphyrin, a naturally occurring porphyrin to which we shall make further reference shortly. These observations serve to link together by direct experimental methods the porphyrins of the hæmoglobins and chlorophylls. Hæmoporphyrin, its methyl ester, and ætioporphyrin are scarcely distinguishable spectroscopically, and their spectra differ only slightly from those of mesoporphyrin and its ester. It is of interest to note that hæmatoporphyrin has a very powerful light-sensitising action. Thus, when injected into animals, it renders them peculiarly sensitive to the noxious effects of ultra-violet irradiation and in other ways increases the response of many tissues to light (ultra-violet) stimuli.

Naturally Occurring Porphyrins.—In recent years H. Fischer and Schumm and their respective schools have devoted considerable attention to the relationships and constitutions of the naturally occurring porphyrins. These may be divided into two groups, according to their solubilities in chloroform :—

Group I.—Insoluble in chloroform.

(1) Coproporphyrin, found in fæces and in yeast as a normal constituent.
(2) Uroporphyrin, found in pathological urine (congenital porphyrinuria).
(3) Turacin, the copper salt of a porphyrin found as a pigment in the feathers of certain birds.

Group II.—Soluble in chloroform.

(1) Kämmerer's porphyrin, extracted from putrefying blood by ether and acetic acid.

(2) Papendieck's porphyrin, found in fæces after a meat meal.

(3) Ooporphyrin, occurring as a pigment in the egg-shells of certain birds, from which it may be extracted by hydrochloric acid.

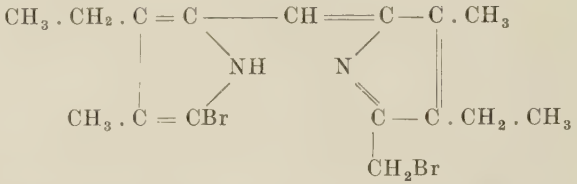
No doubt other porphyrins exist, either in the free state or in such a form as permits of their extraction by means of acid reagents. But there is considerable doubt as to the chemical individuality of the numerous members of the class described from time to time. Recently Fischer has claimed that Kämmerer's porphyrin, ooporphyrin, and the porphyrin obtained by Papendieck by the action of hydrogen sulphide on blood, are identical, and he suggests the general name *protoporphyrin* for this derivative. Since ooporphyrin may be prepared from hæmatoporphyrin on heating the latter in a vacuum, and since in the process two molecules of water are lost, it is suggested that the most probable composition of protoporphyrin is $C_{34}H_{34}O_4N_4$. If these suggestions should prove to be well founded, a considerable and welcome simplification in the classification of the porphyrins would be effected.

Apart from hæmato- and meso-porphyrins, only copro-porphyrin and uroporphyrin have been isolated, analysed, and definitely characterised as chemical individuals. The identity of turacin as the copper salt of uroporphyrin is also fairly well established. Coproporphyrin is formed in the intestine under normal conditions. It is also found as such in normal urine. Even in the majority of pathological conditions which are associated with porphyrinuria it would appear that coproporphyrin is excreted, and only in cases of congenital porphyrinuria is uroporphyrin found. These two porphyrins have very similar spectral properties, and

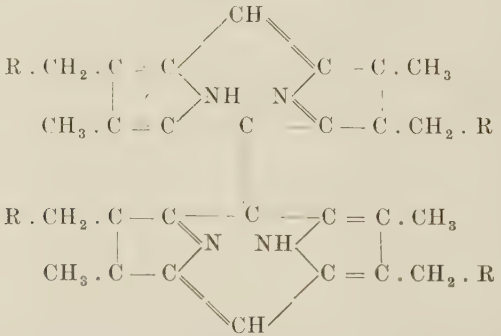
with the heavy metals, especially with iron, they form complexes, having spectroscopic properties very similar to those of hæmin, and forming hæmochromogens with pyridine and other nitrogen compounds. The similarity of the two porphyrins is further borne out by the fact that uroporphyrin is converted into coproporphyrin on being heated with 1 per cent. hydrochloric acid for three hours at 180° to 185°. Coproporphyrin has been detected as a normal constituent of the sera of human subjects, and of horses, oxen, and fowls. It is also found in the fæces of individuals existing on a purely vegetarian diet, and together with Kämmerer's porphyrin it is found in yeast and in beef during sterile autolysis or putrefaction. The presence of porphyrins in yeast, which at first seemed a very puzzling fact, is now readily explained in view of Keilin's discovery in this plant source of the various hæmochromogens which comprise cytochrome.

No general agreement has yet been reached regarding the constitution of the porphyrins, but the following observations represent the best authenticated views. The porphyrins consist of four substituted pyrrol groups linked together through two or four carbon atoms. Ætioporphyrin contains no carboxyl groups and has the formula $C_{31}H_{36}N_4$ (Willstätter) or $C_{32}H_{36 \text{ or } 38}N_4$ (Fischer). Coproporphyrin and uroporphyrin are regarded as being derivatives of ætioporphyrin, in which four and eight carboxyl groups respectively are inserted in the substituent side chains of the pyrrol residues. These two porphyrins therefore have the compositions $C_{36}H_{36 \text{ or } 38}O_8N_4$ (coproporphyrin), and $C_{40}H_{36 \text{ or } 38}O_{16}N_4$ (uroporphyrin), both on the basis of Fischer's formulæ. These views concerning the nature of the porphyrins have recently been substantiated by the brilliant syntheses of ætioporphyrin, ætiohæmin and ætiophyllin by Fischer and Klarer. Starting from cryptopyrrol (3 : ethyl — 4 : 5 — dimethylpyrrol—see p. 322) there is obtained by the action of bromine in cold acetic

acid a compound, $C_{16}H_{20}N_2Br_2$ to which the following formula is assigned :—



This intermediate compound when acted upon by concentrated sulphuric acid yields ætioporphyrin spectroscopically identical with Willstätter's ætioporphyrin from natural sources. By acting upon the synthetic porphyrin with ferric chloride and sodium acetate ætiohæmin is obtained, whilst magnesia and methyl alcoholic potassium hydroxide yield ætiophyllin (see p. 309). On the basis of this synthesis Fischer now assigns the formula shown below on this page to ætioporphyrin ; the formulæ of coproporphyrin and of uroporphyrin may be obtained by inserting suitable substituents in the positions marked **R**.



Fischer and Klarer's suggestion for the formula of ætioporphyrin [R = CH_3], coproporphyrin [R = CH_2 . COOH], and uroporphyrin [R = CH . $(COOH)_2$].

The occurrence of turacin as a pigment in the feathers of certain birds is of some interest, more especially in view of the fact that it contains copper. The functions of this compound as a feather pigment appear to be in marked contrast to

those of hæmocyanin, which is also a copper compound of, presumably, a porphyrin. In 1903 Laidlaw showed that a compound, spectroscopically very similar to turacin, could be obtained from hæmatoporphyrin by treatment with an ammoniacal copper solution. The more recent analyses of Fischer and Hilger suggest very strongly that the pigment is the copper salt of uroporphyrin, with the composition $C_{40}H_{34 \text{ or } 36}O_{16}N_4Cu$. Keilin has recently shown that turacin differs from an iron-porphyrin compound in (*a*) not showing an oxidation or reduction effect, (*b*) not giving a peroxidase reaction, (*c*) not combining with nitrogen compounds.

The Bile Pigments

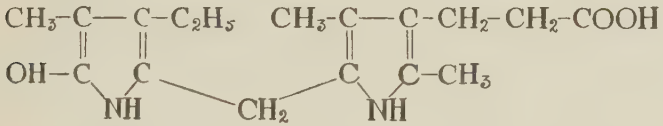
In close chemical relationship to the porphyrins are the bile pigments, and in view of this relationship it is but natural that one should regard the bile pigments as being derived from hæmoglobin or from some related substance. There is ample experimental corroboration of this expectation so far as the intact animal is concerned, but the transformation of hæmoglobin or any of its derivatives into bile pigment has not yet been convincingly demonstrated *in vitro*. It has, however, been claimed that when laked blood is incubated with adrenaline, biliverdin is formed. Brugsch and Pollak have also claimed that by the action of pyrocatechol *in vitro* hæmin and its derivatives are transformed into bilirubin. These claims may be right, but so far they do not seem to have been sufficiently investigated. The evidence upon which is based the statement that bile pigment arises *in vivo* from hæmoglobin may be summarised as follows: (1) Intravascular hæmolysis of red blood cells produced by the injection of distilled water, bile salts, or other hæmolytic agents, leads to the excretion of bilirubin in the urine. (2) The introduction of pure hæmoglobin into the circulation of an animal with a biliary fistula increases the bile pigment excreted by the liver. (3) Pathological conditions in which excessive amounts of hæmoglobin are

liberated in the circulation or in injured tissues, lead to increased excretion of bile pigment or of its derivatives, or to its local formation at the site of the injury. (4) Lastly, the close chemical relationship between the porphyrins of hæmoglobin and bile pigment may be cited as corroborative evidence. Hæmoglobin is the only definitely known source of bile pigment, but the possibility of the formation of the latter from the degradation products of chlorophyll ingested in vegetable matter, and from the muscle pigment cytochrome (myohæmatin), is by no means excluded. It was formerly assumed that the transformation of hæmoglobin into bile pigment took place in the liver, more especially in the epithelial cells of this organ. That the formation of bile pigment may, and does normally, take place outside the liver is now unquestioned, but considerable difference of opinion exists as to the site of the transformation and concerning the mechanism by which it is brought about. Whipple and his associates still attach importance to the epithelial cells of the liver in this respect, whilst Rich regards the hepatic function as being merely an excretory one, namely, the elimination of the bile pigment brought to it by the blood. In addition to the epithelial cells, the Kupffer endothelial cells of the liver have been regarded as playing an important part in the formation of bile pigment, and in recent years considerable importance has been attached to the reticulo-endothelial system generally, to which the Kupffer cells and most phagocytic cells belong. Cells of this type are found in the spleen, bone marrow, lymph glands, blood stream, connective tissue, etc., and any or all of these may be regarded as the locus of the extra-hepatic formation of bile pigment. There is no satisfactory evidence of the presence in any of the tissues mentioned of an enzyme capable of effecting the transformation to bile pigment outside the cell.

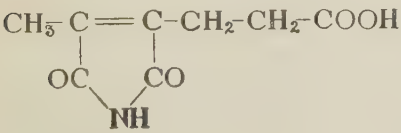
The two most important bile pigments are bilirubin and biliverdin, the compositions of which are expressed in the formulæ $C_{33}H_{36}O_6N_4$ and $C_{33}H_{36}O_8N_4$ respectively. These

formulæ at once indicate a close correspondence with the porphyrins as regards elementary composition, and there is a mass of chemical evidence which bears out this relationship. Much of this evidence is based on the ultimate degradation products obtained from the blood and bile pigments, and a brief reference will be made here to the nature of these products, since they have played an important part in the assigning of constitutional formulæ to the parent substances.

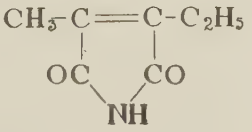
The porphyrins themselves consist of four pyrrol residues, and it is possible to obtain from bilirubin and from hæmin, by reduction with hydriodic acid in glacial acetic acid, a product in which two of these pyrrol groups are still attached to one another. This is known as bilirubinic acid, and Fischer ascribes to it the following constitution :—



Bilirubinic acid, on being oxidised with permanganate, yields xanthobilirubinic acid, in which the hydroxyl group of the parent acid is converted into a ketonic group. On subjecting these derivatives to more drastic chemical treatment various substituted simple pyrrols are obtained, and a large number of such compounds have been isolated and identified in the degradation products of the bile pigments, and of the porphyrins obtained from hæmoglobin, chlorophyll, and their derivatives. Thus by oxidative processes there have been obtained from both blood and bile pigments, hæmatinic acid and methyl ethyl maleinimide, the formulæ of which are given below :—

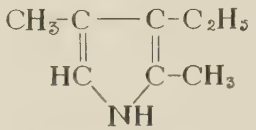


Hæmatinic acid.

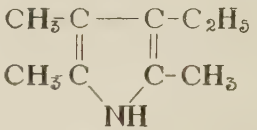


Methyl ethyl maleinimide.

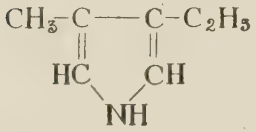
Similarly, by reductive processes the following substituted pyrrol compounds have been obtained, 3 : ethyl-4 : methyl-, 3 : ethyl-4 : 5 : dimethyl-, 2 : 4 : dimethyl-3 : ethyl-, and 2 : 4 :



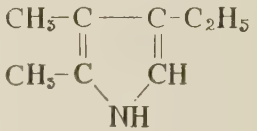
2 : 4 : dimethyl-3 : ethylpyrrol.



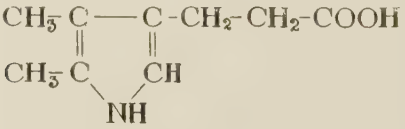
2 : 4 : 5 : trimethyl-3 : ethylpyrrol



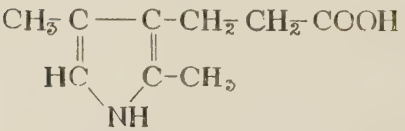
3 : ethyl-4 : methylpyrrol.



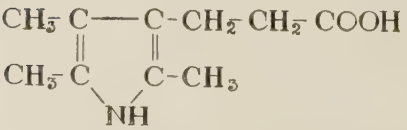
3 : ethyl-4 : 5 : dimethylpyrrol.



4 : 5 : dimethylpyrryl-3 : propionic acid.



2 : 4 : dimethylpyrryl-3 : propionic acid.



2 : 4 : 5 : trimethylpyrryl-3 : propionic acid.

5 : trimethyl-3 : ethylpyrrols, and 4 : 5 : dimethylpyrryl-3 : propionic acid, 2 : 4 : dimethylpyrryl-3 : propionic acid, and 2 : 4 : 5 : trimethylpyrryl-3 : propionic acid. On consulting

the formulæ given opposite the reader will be able to see the relationship of these compounds to the more complex porphyrins and their derivatives, the constitutional formulæ of which have already been given. For a full account of the relationship of each degradation product to the pigments from which it is obtained the reader must consult Fischer's original papers.

On subjecting bilirubin to less drastic reduction than is required for the scission of the individual pyrrol nuclei, mesobilirubin, $C_{33}H_{40}O_6N_4$, and mesobilirubinogen, $C_{33}H_{44}O_6N_4$, are obtained, the former by the use of hydrogen and palladium in alkaline solution, the latter by the action of sodium amalgam. Mesobilirubinogen (urobilinogen) is found in normal bile and urine, and in considerable quantities in certain pathological urines. It is a colourless chromogen which under the action of air and light is transformed into the pigment "urobilin," supposed to be responsible for the strong green fluorescence observed when zinc chloride and ammonia are added to urines containing much of the chromogen. Urobilin is probably identical with the pigment stercobilin, found in the fæces. It would appear that neither pigment is a simple substance, but a mixture of pigments formed from the parent chromogen. It is probably therefore preferable to refer to the urobilin type of pigment rather than to urobilin itself. Presumably a reduction process for which the intestinal bacteria are responsible accounts for the formation of mesobilirubinogen *in vivo*.

Many other bile pigments are known, including choleprasin, biliprasin, bilifuscin, and phylloerythrin, but these are less well defined than those which have been mentioned in the preceding pages. The last-mentioned pigment has the composition $C_{33\text{ or }34}H_{36}O_6N_4$. It occurs in bile not as such, but as a colourless chromogen, from which the pigment is formed on contact with air. A porphyrin isolated by Kéméri, and found in the fæces on both meat and vegetable diets, is probably identical with phylloerythrin.

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CHAPTER XI

THE CHEMICAL BASIS OF SPECIFIC IMMUNOLOGICAL REACTIONS

In the present chapter it is proposed to review certain of the more purely chemical phenomena which seem to underlie the specific nature of immunological reactions. The methods which have hitherto been utilised for the investigation of these reactions have been largely those of the physical chemist or of the biologist, and it has not been found possible to correlate to any extent the findings of either of these groups of workers with the chemical composition or constitution of the reacting bodies so far as these were known. But within comparatively recent years an almost purely chemical series of investigations has yielded results of the greatest importance in contributing to our understanding of the basal mechanisms of immunological reactions, which, in virtue of their marked specificity and great sensitivity, are amongst the most remarkable of biological processes. Before dealing with our main subject we shall devote some brief consideration to the general aspects of immunology which are of importance for a proper appreciation of the immediate problem.

The term "immunological reactions" embraces those specific properties which are not possessed by the normal animal, but which are acquired by the tissues of an animal into which have been introduced intact bacterial cells or "foreign" proteins of bacterial or other origin. It is well known that when a suitable suspension of bacteria such as pneumococci, either alive or dead, is injected in suitable amount into the blood stream of an animal, the latter

acquires after the inoculation an immunity to subsequent infection by a living culture of the same bacteria, and is consequently more or less immune to the specific disease occasioned by their presence. The blood serum of an animal which has acquired such an immunity towards a particular type of pneumococcus gives, when added *in vitro* to a suspension of the same bacteria, certain highly characteristic reactions. In describing these reactions the bacteriologist has evolved a terminology to which we must now refer.

When the serum of an immunised animal, which is referred to as "immune serum," is added in considerable, and sometimes in very high, dilutions to a suspension of the same type of bacteria as was used in the original inoculation, the latter may either "agglutinate" or adhere to one another in clumps, they may be dissolved or "lysed," or if an extract of the bacteria is added to the immune serum a precipitate may be produced, or the mixture may acquire the property of inhibiting the action of fresh serum "complement," the substance present in both normal and immune sera which complements, or assists, certain lytic reactions. These reactions are not obtained when normal blood serum from the same animal species is added to the bacteria or to an extract prepared from them. In the instances mentioned "agglutinins," "lysins," or "precipitins" respectively are said to be present in the immune serum. These hypothetical substances are classified together as "antibodies," whilst the substances which when introduced into the blood stream of the living animal bring about their formation are called "antigens." We shall pass on to a general consideration of the properties of antigens and antibodies.

Antigens

Mention has been made of the fact that both living and dead pneumococci behave as antigens. Antigenic power is therefore not a function possessed only by the living cell,

nor, as we shall see, is it even confined to units possessing a cellular structure. Most proteins, when injected in the same manner as bacteria, induce the formation of specific substances which are found in the serum of the injected animal, and which will cause the mutual precipitation of the constituents of immune serum and the particular protein used for the immunisation when the protein is added to the serum *in vitro*. That is to say, most proteins will behave as antigens, inducing the formation of specific precipitins. In order to exhibit this behaviour the injected protein must, however, be " foreign " to the serum of the animal into which it is injected ; that is, it must not be a normal component of the serum. Now proteins are, of course, present in all bacteria, and we may ascribe the immunological reactions shown by the intact bacteria, or by their disintegration products, to the presence of these proteins. It is, indeed, doubtful if any substances other than proteins possess any antigenic properties, and it seems probable that such properties are possessed by all proteins with the exception of certain members of the group, such as gelatin, which are deficient in aromatic amino acids. On the other hand, proteins such as zein, " incomplete " as regards tryptophane and lysine, but containing considerable amounts of tyrosine, do possess antigenic properties. Proteins which have been irreversibly coagulated by heat or by treatment with alcohol are in general devoid of antigenic activity, but if heating does not lead to loss of solubility, as in the instance of casein, the antigenic action is usually unaffected. Proteins do not lose their antigenic properties on being converted into soluble acid metaproteins, but, on the other hand, the corresponding alkali metaproteins are not antigens. It is known that the peptides and amino acids isolated after alkaline hydrolysis of proteins have in great measure lost their optical activity, whereas those resulting from acid hydrolysis still retain this property. Further, the partly disintegrated products of alkaline proteolysis are not further hydrolysed by suitable proteolytic

enzymes. Dakin has explained these results as being due to enolisation occurring between the CO group of the peptide linkage and the adjoining —CH group, whereby racemisation of the originally optically active amino acid occurs. It would therefore seem possible that loss of antigenic power and of the property of undergoing digestion by enzymes is correlated in some way with racemisation. Fragmentation of the large colloidal protein molecule into smaller molecules proceeds with rapid loss of antigenic power. Moreover, the injection of the total products resulting from the complete hydrolysis of a protein does not produce any antibody formation. The foregoing observations would seem to warrant the conclusion that in order to function as an antigen a substance must be a soluble colloid of the nature of a protein, foreign to the particular animal into which it is injected; apparently it must not be racemised, and it must contain aromatic amino acids. Whilst we must conclude in the absence of definite evidence to the contrary that non-protein substances cannot function as antigens, there is a possibility that certain toxic glucosides may have antigenic properties, and the protein nature of the bacterial toxins which are undoubted antigens has not yet been demonstrated.

Antibodies

The particular response which the organism makes to any given type of antigen varies with the nature of the latter. Thus bacteria and intact cells produce agglutinins and lysins, proteins produce precipitins, and bacterial toxins give rise to antitoxins. Like vitamins and enzymes, antibodies can only be recognised by their effects, and not by what they are. They are probably colloidal, and it is assumed, but it is by

no means certain, that they are proteins. They can be separated from the blood in the globulin fraction of the serum proteins.

The antibodies may be divided into two classes, in the first of which are the agglutinins, precipitins, lysins, and similar substances. There is considerable difference of opinion as to whether the reactions which characterise these substances are to be ascribed to distinct chemical individuals or to different aspects of the reactivity of a single substance. In many cases, after the injection of a single pure protein antigen, the immune serum gives most, or even all, of the well-known immunological reactions. But there are certain discrepancies which do not permit of the acceptance of the simple " unitarian " hypothesis. Agglutination, precipitation, and the lytic reactions depend on alterations in the degree of dispersion of the colloids constituting the substance attacked, but little is known concerning the mechanism of these alterations. In the second class of antibodies are the antitoxins, and these differ from those which we have already mentioned in that they seem to neutralise the toxin for which they are specific by directly combining with the latter in a quantitative manner. When animals of different species are immunised with the same toxin, the antitoxin formed in each case is also the same, but, on the other hand, a marked specificity is shown by the fact that a given antitoxin will only neutralise its own toxin. All attempts to prepare protein-free antitoxins have so far failed.

The Specificity of Immunological Reactions

In recent years the main interest of the biochemist in immunological reactions has centred round the question of their remarkable specificity. Thus precipitins may be obtained which throw down one of the proteins present in the vitreous humour of the eye, but not the other. Again, if a sheep or rabbit be inoculated with the proteins of human

blood serum the antibodies so produced will give little or no reaction with the serum of the ape. Of special interest in their bearing on this question are results which have been obtained with an anti-human serum, and which are quoted by Wells. This serum gave little or no reaction with the blood of mammals other than the primates, whilst excellent reactions were obtained with the blood of primates, as shown by the following table :—

TABLE XX

Anti-human Precipitating Serum

Tested against	Precipitate.
34 specimens human blood . . .	100 per cent.
8 specimens anthropoid blood . . .	100 ,,
36 specimens blood from common monkeys .	92 ,,
13 specimens blood from capuchins and spider monkeys	78 ,,
4 specimens blood from marmosets . .	58 ,,
2 specimens blood from lemurs . . .	0 ,,

(Quoted from Wells.)

This specificity is peculiarly striking in the instance of the antibodies produced by bacteria, for not only is the serum of a patient suffering from pneumonia specific for pneumococcus alone and quite inactive to typhoid bacilli, and *vice versâ*, but within one species of pathogenic organism it would appear that commonly various types exist which are immunologically quite distinct and characteristic. To find an adequate chemical basis for the multiplicity of these specific reactions might well be regarded as beyond the powers of even the most sanguine biochemist. Nevertheless, recent developments in this field have yielded results of such a promising nature that considerable advances in the interpretation of the mechanism of these phenomena have already been made.

Up till the present time the organism which has been most extensively investigated from the standpoint with which we are concerned is the pneumococcus. Pneumococci obtained from different sources frequently present individual characteristics, and it is possible to divide them into three well-defined strains, which are referred to as Types I., II., and III. Certain sub-types have been recognised, but these need not enter into our present considerations. If we are dealing with Type I., the immune serum or antiserum produced on inoculation of Type I. pneumococci is said to be an " homologous antiserum," whilst serum produced by the other types is said to be " heterologous."

In 1917 Dochez and Avery showed that in bacteria-free filtrates of young cultures of pneumococcus there was present a specifically reacting substance which precipitated with antipneumococcal serum of homologous type. The same substance was also found in the blood and urine of animals infected with pneumococcus. In a study of the urine of 112 cases of lobar pneumonia it was found that in 62·5 per cent. of the cases attributable to pneumococcus of Types I., II., or III. a substance was excreted in the urine which reacted specifically with homologous antipneumococcal serum. Later Zinsser and Parker isolated what they called " residue antigens " from alkaline extracts of pulverised bacteria of several varieties, including pneumococci. These residue antigens were resistant to acid and to heat, did not appear to contain protein, and were specifically precipitable by homologous antisera. Despite this last-mentioned fact, these residue antigens did not produce antibodies when injected into animals. They were therefore, strictly speaking, not antigens. At the time of its discovery Dochez and Avery found that their specific substance is not destroyed by boiling. It is readily soluble in water, is precipitated by acetone, alcohol, ether, and by dialysed iron, does not diffuse through parchment, and is not affected by treatment with a proteolytic enzyme such as trypsin. In view of these properties

Heidelberger and Avery regarded this substance as well suited for the commencement of their studies of the relation between bacterial specificity and chemical constitution.

In 1923 the first results of this investigation were published. The paper, together with subsequent publications, dealt with two of the cellular constituents of the pneumococcus. One of these is precipitated from solution by dilute acetic acid, and is referred to as bacterial nucleoprotein, although it probably consists of a mixture of proteins. The other constituent, the soluble specific substance, is now known to be a carbohydrate of the polysaccharide type. Heidelberger and Avery point out that these two substances probably do not comprise the whole antigenic mosaic of the cell, but, as we shall see, they have served to elucidate some of the fundamental aspects of immunological reactions. Investigations have been made of the behaviour of the nucleoprotein and of the soluble specific carbohydrate, both as individual substances and as they exist together in the intact cell. Each type of pneumococcus yields a nucleoprotein possessing antigenic properties which on injection into rabbits produces an antinucleoprotein serum. But these antinucleoprotein sera are not type-specific, so that the antiserum produced by inoculation of Type I. pneumococci will react equally well with the proteins of Type II. and Type III. as with those of Type I. cocci. The same nucleoprotein would therefore seem to be present in all types. It should be noted that these antiprotein sera will not agglutinate type-specific strains of pneumococcus, and will not precipitate solutions of the soluble specific carbohydrate (see Fig. 37). On the other hand, the carbohydrate is different in each type of pneumococcus. The following quotation from one of the papers of Avery and Heidelberger will serve to emphasise these points : " Pneumococcus is an encapsulated organism, and there are grounds for the belief that the ectoplasmic layer of the cell is composed of carbohydrate material which is identical in all its biological

characters with the type-specific substance of pneumococcus. On the other hand, the endoplasm, or somatic substance, consists largely of protein which is species and not type specific. The protein is possessed in common by all pneumococci, while the carbohydrate is chemically distinct and serologically specific for each of the three fixed types. The cell, therefore, may be conceived of as so constituted that there is disposed at its periphery a highly reactive substance upon which the type specificity depends." The fundamental distinction, then, between the two substances is that the nucleoprotein is species-specific and antigenic, whereas the carbohydrate is type-specific, but non-antigenic.

The Chemical Nature of the Specific Carbohydrate Substances

The method adopted for the separation of the soluble specific substances of pneumococcus is naturally long and tedious, and the yield of final product is, as might be anticipated, small, some 2 to 3 grams from 300 to 325 litres of culture. Briefly, the separation of the Type I. specific substance involves the following operations. The cultures are concentrated in 2-litre lots and precipitated by the addition of alcohol. On centrifuging three layers are obtained, the middle one of which contains the specific substance. This layer is dissolved in water, and various impurities are precipitated and removed by acidifying strongly with hydrochloric acid. After repeated centrifuging at high speed the crude specific substance is thrown down by the addition of chilled alcohol, separated by centrifuging, and washed with 0·5 N acetic acid to remove glycogen and erythrodextrin. The product is then dissolved in sodium hydroxide, centrifuged, and precipitated by the addition of alcohol in the presence of sodium acetate. The subsequent lengthy purification processes involve redissolving the substance, repeated precipitations, filtration through Berkfeld candles, precipitation at the isoelectric point, dialysis in collodion sacs, and washing with acetone. Although no claim is made that the

carbohydrates obtained in the above and in similar ways are pure chemical individuals, the various preparations show a marked constancy in their composition and in their physical and chemical properties, and every care seems to have been taken to guard against the possibility that the active substances might be merely adsorbed impurities. The chemical characteristics of the specific substances sharply differentiate the three carbohydrates. These characteristics are given in Table XXI, which is constructed from the average results and analyses recorded by Heidelberger, Goebel, and Avery.

TABLE XXI

Chemical Characteristics of the Specific Carbohydrates separated from Pneumococcus Types I., II., and III.

Type.	Percentage Composition.*					Acid Equivalent.	Optical Rotation.	Reducing Sugars on Hydrolysis.
	C.	H.	N.	Amino N.	Reducing Sugar on Hydrolysis.			
I.	43·3	5·65	4·8	2·55	28·5	—	+ 300°	Galacturonic acid, amino sugar.
II.	45·8	6·4	0·0	—	68·3	1,175	+ 66·4°	Glucose, glycuronic acid.
III.	42·7	5·3	0·0	—	73·2	343	− 33·0°	Glucose, glycuronic acid.

* A polysaccharide of the composition ($C_6H_{10}O_5$) requires C = 44·4 and H = 6·2 per cent.

Limiting Dilutions in which Precipitation with homologous Antipneumococcus serum is obtained

Type.	Dilution.
I.	1 : 4,000,000 to 1 : 8,000,000
II.	1 : 2,000,000 to 1 : 6,000,000
III.	1 : 5,000,000 to 1 : 6,000,000

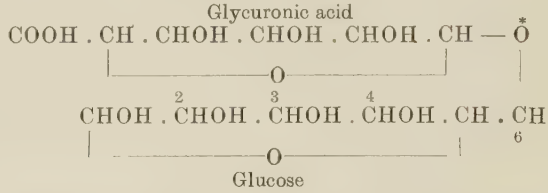
From the above table it will be seen that the Type I. substance differs from the other two in containing nitrogen.

Despite this, it gives none of the usual protein colour tests. On being treated with nitrous acid it loses one-half of its nitrogen, so that this proportion is presumably amino nitrogen. At the same time reducing sugars appear, and the specific action is lost. Similar treatment with nitrous acid of the Types II. and III. substances, which do not contain nitrogen, does not result in loss of their specific activity. One may therefore infer that at least one-half of the nitrogen of substance I. is an integral part of the compound and directly associated with its specificity. From the fact that the carbon and hydrogen content closely approximate to those required for a carbohydrate it is inferred that there is present in this substance a nitrogenous sugar of the type of glucosamine. Oxidation with nitric acid yields mucic acid, which indicates the presence of galactose, or it may be that this acid arises from oxidation of the galacturonic acid which is also thought to be present. Type I. substance is amphoteric.

The soluble specific substance obtained from Type II. pneumococcus is a nitrogen-free polysaccharide made up of glycuronic acid and glucose units. Type II. substance has been acetylated with pyridine and acetic anhydride. The resulting acetyl compound can be hydrolysed, and the original specific substance may be recovered. Carbohydrate II. is weakly acidic.

The Type III. substance is similar to Type II., but is sharply distinguished from the latter in being *lævo*-rotatory. Like substance II., it is also acidic. Heidelberger and Goebel have recently initiated a more detailed study of the chemical nature of the Type III. carbohydrate, and conclude that the specific substance is a polymeride of a glucose-glycuronic acid. Thus intensive hydrolysis of the specific carbohydrate using sulphuric acid yielded an aldobionic acid of the formula $C_{12}H_{20}O_{12}$ having $[\alpha]_D + 10{\cdot}0^\circ$ in water, and possessing one-half of the reducing power of glucose. Hydrolysis of this aldobionic acid with hydrobromic acid containing bromine yielded saccharic acid. Further oxida-

tion of the aldobionic acid with barium hypoiodite gave a dicarboxylic acid, $C_{10}H_{18}O_9$ $(COOH)_2$, and this new acid yielded the same amount of furfuraldehyde as the original aldobionic acid. On these findings the following structural formula may be ascribed to the aldobionic acid which forms the unit of the Type III. carbohydrate :—



The oxygen atom marked with a star may be attached to any one of the carbon atoms 2, 3, 4, or 6 of the glucose residue.

Immunological Reactions of the Specific Pneumococcus Carbohydrates

The specific carbohydrates which we have just described are not antigens. They do not evoke any antibody response when they are injected into animals. Thus if a solution of one of them be injected into a rabbit, the serum of that rabbit does not acquire the property of forming a precipitate when it is added to a solution of the carbohydrate used in the injection. On the other hand, the carbohydrates do react specifically with the antibody in the homologous sera produced by immunisation with the corresponding intact bacteria. Especially significant from our present standpoint is the fact that, although each specific carbohydrate precipitates homologous antisera in extreme dilutions ranging up to 1 : 8,000,000, the Type I. carbohydrate will not precipitate Type II. or Type III. antisera, but reacts specifically with Type I. alone. Similarly, Type II. and Type III. carbohydrates are equally specific. At much higher concentrations of the carbohydrates slight and indefinite cross-precipitations

may be obtained, but there is no question of the essential specificity of these substances. It has been shown that the carbohydrate is present in the precipitates and may be recovered in a practically unchanged condition after tryptic digestion and used again.

The observation that these carbohydrates are non-antigenic when they are dissociated from the other cellular constituents, and are capable of inciting specific antibody formation only when they are present in the intact cell, leads Avery and Heidelberger to conclude that in the intact pneumococcus the carbohydrate is probably present in combination with some other substance which confers upon it antigenic properties. In these authors' own words, "Immunisation with intact bacteria containing this carbohydrate complex elicits antibodies which not only agglutinate the formed cells, but precipitate solutions of the carbohydrate isolated from pneumococci of the homologous type. How the specific carbohydrate is combined in the cell, whether with protein or some other constituent, is not yet clear, but it is evident that the compound thus formed is the dominant and essential antigen of the cell, and the one responsible for type specificity." Strong support for the view that some definite combination between carbohydrate and some other cell constituent must exist in the intact cell is afforded by the observation that on morphological dissolution of the bacteria, either by freezing and subsequent thawing or by the lytic action of bile, the solution obtained, although it contains all the carbohydrate and protein substances of the original whole bacterium, does not induce the formation of type-specific antibodies. Antiprotein sera are obtained which are identical in the cases of all three types of cocci, and these will not react with the homologous carbohydrates. When the cell is ruptured its constituents pass into solution, the linkage between the carbohydrate and the other constituent is broken, and the free carbohydrate fraction, although still retaining the property of specific union with

its type antibody, is no longer capable of inciting the formation of this same immune body. It would appear that the complex, if such it is, is not easily re-formed once it is destroyed, since sera prepared from filtered solutions of disintegrated cells free from formed elements fail to show any of the dominant type-specific properties which characterise sera obtained by immunisation with whole bacteria (compare A and C, Fig. 37). Avery and Heidelberger conclude that "morphological integrity of the bacterial cell, therefore, is requisite for the expression of its full antigenic power."

When intact pneumococci are injected into a rabbit specific agglutinins are formed for that type of pneumococcus, and also specific precipitins for the corresponding carbohydrate. In addition some varying amounts of nucleoprotein precipitins are formed, but these, as already mentioned, are not type-specific. There is some doubt as to whether any nucleoprotein precipitin is formed as a result of the injection of the intact pneumococci as such. It is the opinion of Avery and Heidelberger that the nucleoprotein precipitins which usually appear are due to the fact that in most suspensions of pneumococci as used for injection a certain amount of dissolution of the bacteria can scarcely be avoided, and hence a small quantity of free nucleoprotein is usually present. Even if this dissolution were avoided, disintegration of some of the injected bacteria would occur after the inoculation.

The reader will see from the foregoing that, whilst it is not yet possible to arrive at a definite conclusion with regard to the form in which the carbohydrate exists in the intact cell, it seems at present justifiable to suppose that it is associated in some way with the bacterial nucleoprotein. According to this supposition, the scheme given in Fig. 37, which is reproduced from one of Avery and Heidelberger's papers, and which is purely symbolic, will serve to visualise the correlation between the cell constituents and their respective antibodies.

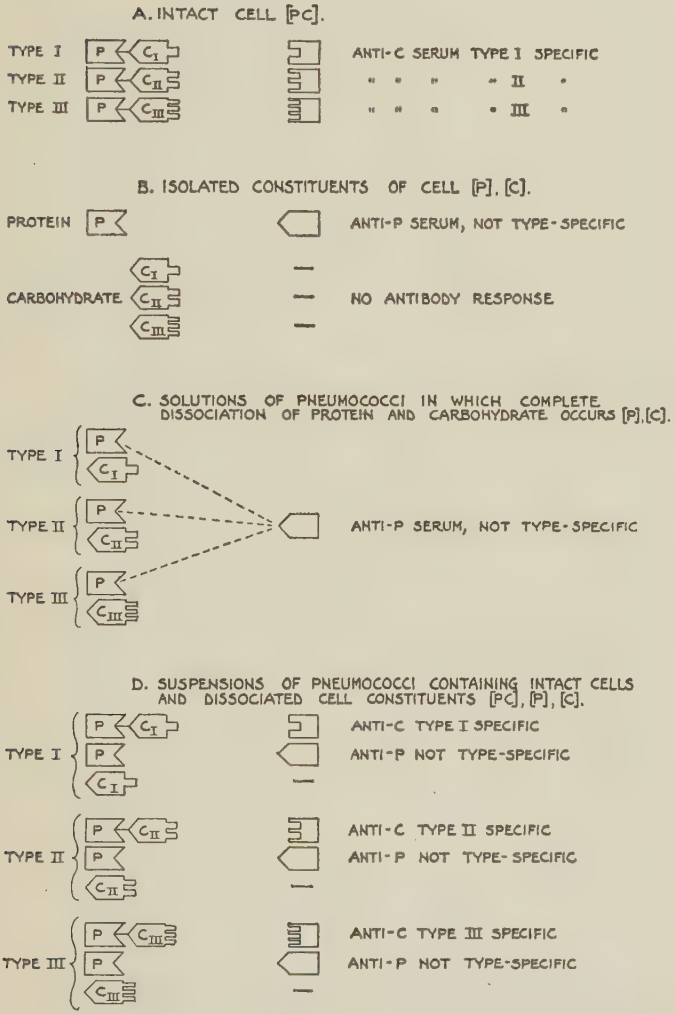

A. INTACT CELL [PC].
TYPE I
TYPE II
TYPE III
ANTI-C SERUM TYPE I SPECIFIC
B. ISOLATED CONSTITUENTS OF CELL [P], [C].
PROTEIN
ANTI-P SERUM, NOT TYPE-SPECIFIC
CARBOHYDRATE
NO ANTIBODY RESPONSE
C. SOLUTIONS OF PNEUMOCOCCI IN WHICH COMPLETE DISSOCIATION OF PROTEIN AND CARBOHYDRATE OCCURS [P], [C].
ANTI-P SERUM, NOT TYPE-SPECIFIC
D. SUSPENSIONS OF PNEUMOCOCCI CONTAINING INTACT CELLS AND DISSOCIATED CELL CONSTITUENTS [PC], [P], [C].
ANTI-C TYPE I SPECIFIC
ANTI-P NOT TYPE-SPECIFIC
ANTI-C TYPE II SPECIFIC
ANTI-P NOT TYPE-SPECIFIC
ANTI-C TYPE III SPECIFIC
ANTI-P NOT TYPE-SPECIFIC

FIG. 37.—Immunological relationship of the cell constituents of Pneumococcus of Types I., II., and III. C = carbohydrate, P = protein. (After Heidelberger and Avery.)

Specific Substances from other Micro-organisms

The nature of the results obtained in the case of the various types of pneumococcus naturally led to the investigation of the possible occurrence of soluble specific substances in other micro-organisms. Following up the results of Zinsser and Parker on the preparation of the so-called residue antigens, Mueller and Tomesik were able to show that from a totally different type of micro-organism, namely, yeast, it was possible to isolate a complex carbohydrate which was apparently identical with the "yeast gum" described by earlier workers, and which gave precipitin tests with immune yeast sera in dilutions up to 1 : 400,000, and negative results with the serum of normal controls. Like the pneumococcus carbohydrates, the specific substance isolated from yeast completely failed to invoke any antibody response when injected into animals. The specific rotation was of the order + 90°, and small amounts of nitrogen and phosphorus were still present in the purest preparations obtained.

In extending their work on pneumococcus, Heidelberger, Goebel, and Avery decided to investigate Friedländer's bacillus, presumably on account of its voluminous capsule. It will be remembered that the capsule of pneumococcus was supposed to be composed almost wholly of the specific carbohydrate substance, and on analogy it was therefore reasonable to suppose that a bacillus with a prominent capsule offered suitable material for the isolation of a soluble specific substance should such be present. Friedländer's bacillus is quite distinct from pneumococcus both in form and in function, although it is sometimes associated with it in the production of pneumonia. In one respect the two bacteria resemble one another, since both exist in the form of a number of distinct immunological types, but in the case of Friedländer's bacillus these have not been worked out so completely as have the types of pneumococcus. Work was commenced on a strain (E) isolated from a chance

infection of a laboratory guinea-pig. Using the methods already described, a carbohydrate was isolated which had the following characteristics. It contained no nitrogen, was weakly acidic, having an acid equivalent of 695, and had a specific rotation of + 100°. The principal hexose component of this new polysaccharide proved to be glucose, which was identified by the isolation of the osazone and by the formation of saccharic acid on oxidation. The presence of glycuronic acid was also detected. The analytical figures showed C = 44·6 and H = 6·1 per cent., in very close agreement with the figures required for a polysaccharide of the composition $(C_6H_{10}O_5)_n$. Goebel has recently shown that on hydrolysis this specific carbohydrate, like that isolated from Type III. pneumococcus, yields an aldobionic acid $C_{12}H_{20}O_{12}$, which is a glucosidic compound of glucose and glycuronic acid of the same type as, and isomeric with, the acid obtained in the same way from the Type III. carbohydrate (see p. 336). It is probable that another unidentified sugar acid enters into the make-up of the Friedländer polysaccharide in addition to the above described aldobionic acid and a molecule of glucose.

A scrutiny of the properties of the Friedländer substance reveals a striking resemblance to those of the pneumococcus Type II. substance. The agreement, while not absolute, suggested to Heidelberger and Avery the experiment of cross-precipitation tests, and the remarkable fact emerged that the Friedländer substance gave a precipitate with the anti-pneumococcus Type II. serum in dilutions up to 1 : 2,000,000. Conversely, pneumococcus Type II. substance gave a precipitate with anti-Friedländer (E) serum. On the other hand, no trace of reaction was observed with the Friedländer substance and Type I. or Type III. antipneumococcus sera. It must not, however, be supposed that the two substances are identical; the rotations, acid equivalents, and certain precipitation reactions, even granted that neither substance has been obtained pure, are too divergent to permit of this

possibility. Nevertheless their marked immunological similarity renders reasonable the assumption that both substances contain in a portion of their complex molecule the same or a closely similar configuration of atoms. This remarkable example of what is called "heterogenetic specificity" strikingly illustrates the real importance of the chemical nature of the carbohydrate in the immunological reactions. It may also be mentioned here that these cross-reactions also apply to the intact organisms. Thus mice could be protected against inoculation with Type II. pneumococcus by means of a previous injection of anti-Friedländer (E) serum just as well as, and possibly even better than, by injection of anti-pneumococcus Type II. serum.

Laidlaw and Dudley have made a further interesting contribution to the problem of these substances with specific immunological reactions. These investigators have extracted from tubercle bacilli (Human Type, Strain 100) a carbohydrate possessing properties closely analogous to those of the pneumococcus carbohydrates. After treating the pulverised bacilli so as to remove the bacterial residue, proteins, and tuberculinic acid (a nucleic acid), a material was obtained which proved to be largely carbohydrate in nature, and which could be separated into glycogen and another carbohydrate isolated in the form of a gum and finally as a white powder. Solutions of the latter gave in high dilutions (up to 1 : 6,400,000) a precipitate with immune horse serum, whilst with normal horse serum no precipitation was obtained in any concentration of the precipitating substance. This specific carbohydrate from tubercle bacilli gives no colour reaction with iodine, does not reduce alkaline copper solutions, nor does it contain nitrogen or sulphur. On hydrolysis pentoses and methyl pentoses were detected. No proof of the presence of glucose or of other hexoses was obtained. Dissolved in 2·5 per cent. sulphuric acid, the substance showed a specific rotation of + 67°. On heating the rotation diminished to zero, and from the neutralised solution

a small quantity of an unidentified crystalline osazone was obtained. Mueller, working with filtered culture media in which tubercle bacilli had grown, has recently reported the isolation of a similar carbohydrate, containing at least 55 per cent. of pentose constituents, which gives specific precipitin reactions in high dilution in the presence of homologous immune sera.

Still another case in point is provided by the non-hæmolytic *Streptococcus viridans*. The immunological reactions of two of the cell constituents of this micro-organism have been studied by Lancefield. At the time of writing this investigation is incomplete, but the results, so far as they have been recorded, bear out the analogy to the pneumococcus results in a striking way. One of the constituents, the so-called nucleoprotein, is relatively non-specific and gives rise to an antibody which shows group reactions with nucleoproteins of related species. The other constituent is non-protein, as indicated by qualitative chemical tests, and is very probably a carbohydrate. Although the latter is highly reactive with the specific antibodies produced by immunisation with the intact bacterial cell, in the purest condition in which it has been obtained it is itself non-antigenic. It will be remembered that Heidelberger and Avery and their associates isolated from the three types of pneumococcus one and the same nucleoprotein, identical in immunological relationships, and it will be seen that a similar non-specific protein is obtained from *Streptococcus viridans*. These proteins constitute a highly interesting group of substances, but since from the chemical point of view they have not yielded to purification to the same extent as the carbohydrates, our information about them is still somewhat indefinite. They seem to exist constantly along with the carbohydrates, and it would appear that they too play an intimate *rôle* in immunological phenomena.

Finally, in closing this account of a most promising and suggestive development in biochemistry, we may mention

the recent work of Landsteiner and Levine on *Vibrio cholerœ*. Here again this organism has yielded an antigenic protein, and a protein-free product active in the precipitin test, but almost completely devoid of antigenic activity. The latter substance yields on hydrolysis a considerable quantity of carbohydrate. It also contains nitrogen and phosphorus. These may be present as impurities in a complex carbohydrate of the type described by Avery and Heidelberger, or it may be that the specific substance of *V. cholerœ* has a more intricate structure than those of the pneumococci.

From this brief sketch of these recent developments in immunology it is apparent that there is already a substantial bulk of evidence to show that these peculiar carbohydrates are of fundamental importance in what have been regarded up till the present time as obscure immunological reactions ; more especially are they of importance in determining one of their most interesting features, namely, their specificity. That Nature should have selected carbohydrates for this *rôle* is perhaps not surprising when we bear in mind the numerous possible ways in which two simple sugar molecules may be linked together, each one distinct from the others in stereochemical configuration. Thus there are at least twenty-eight ways in which two molecules of *d*-glucose may combine with one another, and when we consider the possibilities offered by two different sugars these become still greater. In comparison the possible modes of combination of two amino acids are very limited, and although numerous isomeric peptides could result from the combination of, let us say, twelve amino acids, this number becomes insignificant when compared with the number of possible carbohydrates obtainable from twelve molecules of glucose—somewhere in the order of 10^{18} !

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CHAPTER XII

CHEMOTHERAPY

Chemotherapy is that branch of pharmacology which is concerned with the combating of infectious diseases of bacterial or protozöal origin by the use of internal antiseptics, that is to say, of antiseptics which must be introduced into the blood stream of the living infected host in order to produce their specific effects. One may also regard the term "chemotherapy" as embracing the use of external antiseptics, many of which are identical with or closely related to the internal antiseptics. The whole field is one in which the endeavours of the synthetic organic chemist have recently led to some striking successes.

Chemotherapy of Infectious Diseases

The practice of combating certain maladies of an infectious nature with specific organic or inorganic medicaments is a very old one, dating from times long before the causation of the disease or the rationale of its treatment were known, but, being based purely on empiricism, it necessarily remained more of an art than a science until comparatively recent years. Modern developments in this branch of biochemistry have their origin in the early observations (1890) of Ehrlich on the selective staining action of certain organic dyestuffs towards various living cells and pathogenic organisms, and the attempt to combine with this property a toxic action which would kill the parasite without harming the infected host. The aim has been, then, to obtain a preparation of maximal toxicity to the parasite (parasitotropic), and of

minimal toxicity to the tissues of the host (organotropic), so that if C be the curative dose which kills all the parasites, and T the maximal dose which the host will tolerate without suffering injury, the ratio C/T should be small if the preparation is to meet with success. Much of Ehrlich's later work was based on the view that it was possible to select compounds for which the particular organism whose end they were designed to encompass had a special affinity, resident in certain " receptor " groups which became linked to some special atomic grouping in the antiseptic ; combination being established, the vital activity of the invading parasite was then so lowered that it ceased to produce its toxins and died, or the natural defensive mechanisms of the host's tissues attained the upper hand and achieved the same end. This simple theory of direct affinity between parasite and antiseptic has proved exceedingly useful in a purely practical way, and has led to the elaboration of many new and more toxic compounds of higher selectivity for given infections, but, as Dale points out, it is becoming increasingly apparent that such a simple hypothesis is no longer tenable. Thus, whilst in certain cases the mechanism postulated by Ehrlich would still seem to furnish the simplest explanation, there are many examples of therapeutic agents which are toxic to organisms *in vitro* or in cultures, but which are entirely ineffective when introduced into the host's blood stream, and conversely many of the most powerful antiseptics *in vivo* prove to be those which are quite inactive, or practically so, *in vitro*. Evidence, too, is available which shows that certain dyestuffs of a powerfully specific antiseptic nature have a much greater affinity for the tissues of the host than for the isolated parasites *in vitro*, and, again, at least one case is known in which the antiseptic agent confers on the host a specific immunity to the infection not unlike that produced by protein antigens to bacterial toxins. Obviously a simple theory of direct affinity must be extensively modified to explain these cases.

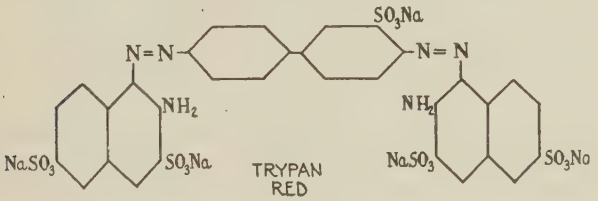
The diseases which we are considering fall into one or other of two main classes according as they are caused by the presence in the infected animal of a foreign protozöon, or of a bacterium, and it is to be noted that chemotherapeutical methods have proved very much more effective in combating the former type of infection than the latter.

Chemotherapy of Protozöal Diseases

It is in this group that the most striking successes of chemotherapy have been achieved. Four types of antiseptics have proved useful; these are: (1) Organic dyestuffs; (2) organic compounds related to dyestuffs, but not necessarily dyestuffs or even coloured; (3) organo-metallic compounds, chiefly those of arsenic, and to a less extent derivatives of antimony, bismuth, mercury, and certain rare metals; (4) alkaloids, chiefly those occurring in cinchona bark or ipecacuanha root, or artificially prepared derivatives of these. The diseases which have proved most amenable to treatment by these specifics are trypanosomiasis, spirochætal, malarial, and amœbic infections, many characteristically those prevalent in, or acquired in, tropical countries.

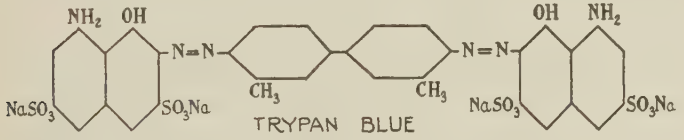
(1) **Dyestuffs.**—One of the earliest observations in this field is due to R. Koch, who noted the antiseptic action of Malachite green, and in 1891 Ehrlich and Guttman investigated the possibility of using Methylene blue, which has a strong staining action on the malarial parasite, as a specific curative agent for that infection. Following on Koch's early observations on Malachite green, many other dyestuffs of the triphenylmethane series—Parafuchsin, Tryparosan, Methyl violet, Methyl green, Aniline blue—have been used, but, owing to their deleterious local action on tissues, they have not been extensively employed. Later investigations led to the use of dyestuffs obtained by tetrazotising benzidine or tolidine and coupling the product of this reaction with various substituted naphthylamines or amino naphthols;

thus Trypan red and Trypan blue have proved of considerable use. The former dyestuff illustrates the point, already



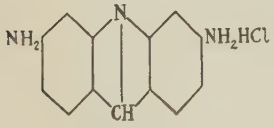
TRYPAN RED

mentioned, of an antiseptic which is inactive *in vitro*, yet proving effective against certain trypanosomes (*T. equinum*) in mice, and demonstrates the further point of a marked

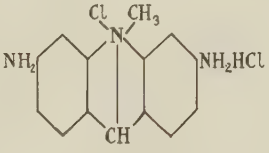


TRYPAN BLUE

specificity, being inactive against *T. equinum* in the rat, or against the trypanosome of Nagana (*T. brucei*) in the mouse. Trypan blue is effective against the last-mentioned infection, and has been used in curing dogs and cattle of a parasitic



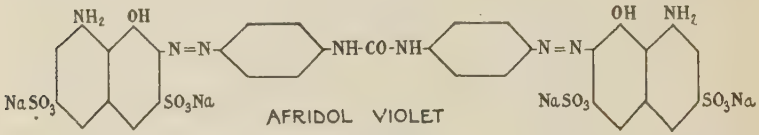
PROFLAVINE



TRYPAFLAVINE

infection of the red blood cells (*Piroplasma*). Of more recent interest is the introduction of dyestuffs of the acridine series ; thus Proflavine and Trypaflavine (Acriflavine) have proved of some use in trypanosome infections, but are much more useful on account of their anti-bacterial action or as external

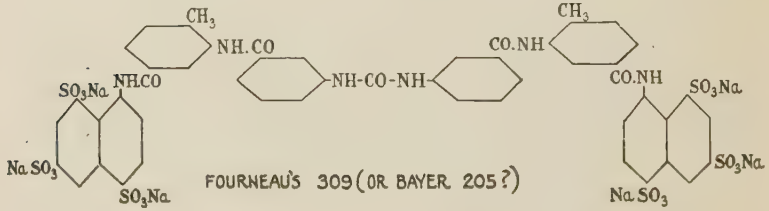
antiseptics. Browning has, indeed, shown them to possess the useful property of augmenting their bactericidal action in the presence of organic media such as blood sera. The dyestuff Afridol violet, which is very similar in constitution



AFRIDOL VIOLET

to Trypan blue, but is obtained by using tetrazotised diaminodiphenylurea in place of tolidine, is of considerable interest, not on account of its own practical value, which is but slight, but because it is a complex substituted urea, and as such forms a link between the trypanocidal dyestuffs and the second group of compounds already mentioned.

(2) Organic Compounds related to Dyestuffs.—One of the most striking advances in the chemotherapy of trypanosomiasis has resulted from the introduction, not of a new dyestuff, but of a related compound which is devoid both of dyeing properties and of colour. This is known as "*Bayer* 205," and has been introduced by the Bayer Company, who have not, however, disclosed its constitution. It would seem to possess a much more marked action against trypanosomes than any of the numerous dyestuffs previously used. It has recently been extensively studied by Fourneau and his collaborators, and these workers have succeeded in synthesising a compound to which the serial number 309 is given, and which has the constitution shown :—



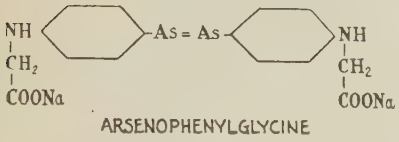
FOURNEAU'S 309 (OR BAYER 205?)

This new compound 309 has trypanocidal and other properties very closely resembling, if not identical with, Bayer 205. Moreover, Fourneau and his co-workers have prepared numerous substances of a nature closely similar to that of 309, and report that a very slight constitutional change completely suppresses the trypanocidal action. On comparing the formula of 309 with Afridol violet or Trypan blue it will be seen that the former differs from the two latter in the absence of diazo linkings (accounting for its lack of colour) and in possessing a chain of —NH . CO— groupings which confers on it a certain constitutional similarity to the peptides. Bayer 205 has no action on trypanosomes *in vitro*, and one of its most remarkable properties is the capacity of a single injection not merely to cure the infection, but to confer on the animal an immunity to subsequent trypanosome infections which may last for weeks, or even months. Following the initial successful use of Bayer 205 in animals, it has been applied to the cure of trypanosomiasis in man, and is reported to have cured completely advanced cases of infection with *T. gambiense* (sleeping sickness) and *T. rhodesiense.* It is just possible that early estimates of the potency of this new specific applied to human trypanosomiasis are a little too optimistic. It would appear that Bayer 205 is slow in its action and persists in the blood and tissues for some time. The sera of patients who have been treated with the drug several weeks previous to the withdrawal of the sample of blood under examination are found to possess a trypanocidal action, which, however, Luengo and de Buen ascribe not solely to the drug still present in the blood, but also to the products resulting from the destruction of the trypanosomes. Most investigators report that Bayer 205 has a tendency to produce a more or less persistent nephritis. In any case, the evolution of this type of colourless, complex, substituted urea—Bayer 205 or Fourneau's 309—from the tetrazotised benzidine dyestuffs is an interesting chapter in therapeutics, and represents a very noteworthy

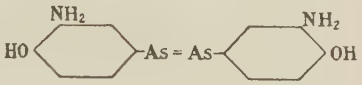
advance which presents many possibilities of future development.

(3) Organo-metallic Compounds.—The chief of these are the arsenicals, and they have attained great importance in combating trypanosome and spirochætal infections. The use of arsenic derivatives in this connection dates from the end of last century, when arsenious acid was used as a remedy for certain parasitic infections in animals. But the first complex organic arsenical to achieve success—and it still remains in use to-day—was atoxyl, which was shown by Ehrlich and Bertheim in 1907 to be the sodium salt of arsanilic acid (*p*-aminophenylarsinic acid). Atoxyl has proved a useful starting substance for the preparation of many arsenicals and for the general study of arsenic therapy, and is itself still one of the most effective remedies for sleeping sickness. Atoxyl is practically devoid of action on trypanosomes *in vitro*, and Ehrlich formed the theory that it became reduced *in vivo* to an actively toxic modification. Acting on this view, he prepared a number of reduced arsenicals and was able to demonstrate their increased toxicity. Thus he found that *p*-hydroxyphenylarsinic acid, effective *in vitro* against trypanosomes in a dilution of 1/20, on reduction yields *p*-hydroxyphenylarsenious oxide, which is effective at a dilution of 1/1,000,000. A similar relation was found to hold between atoxyl and its reduction product, *p*-aminophenylarsenious oxide, but, unfortunately, the increased toxicity of the reduced compound to the parasite is accompanied by a corresponding increase in toxicity to the host, so that the tolerated dose diminishes ; hence the impracticability of direct injection of arsenoxides in chemotherapy. The general fact emerged from these researches that derivatives in which the arsenic is present in a trivalent condition are much more toxic to spirochætes than compounds containing pentavalent arsenic, and this led to the preparation of arseno derivatives of the general type, R—As = As—R, of which arsenophenylglycine and the well-known salvarsan

or " 606 " (Ehrlich and Bertheim, 1912) and their derivatives have proved most useful. It should be noted that these



ARSENOPHENYLGLYCINE

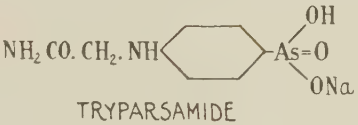


SALVARSAN

compounds represent a further stage in the reduction process :—

$$R—As\overset{OH}{\underset{OH}{=}}O \longrightarrow R—As=O \longrightarrow R—As=As—R$$

Arsenophenylglycine is very effective against trypanosome infections in animals, but when applied to the cure of sleeping sickness in man results of a less promising nature have been obtained. Here the somewhat anomalous fact may be noted that the most effective arsenical yet produced for the treatment of human sleeping sickness is tryparsamide, in which the arsenic is pentavalent, an indication that, fruitful as



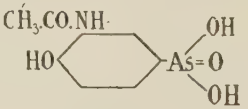
TRYPARSAMIDE

Ehrlich's reduction theory proved in producing results of practical value, it does not represent the whole truth. In any case, tryparsamide and Bayer 205 constitute our best defences against the attacks of *T. gambiense*.

Salvarsan remains one of the greatest discoveries of this branch of therapeutics, being effective in both spirochætal and trypanosome infections, although its widespread use as an anti-syphilitic agent in the former capacity rather obscures its usefulness in the latter. As in the case of the atoxyl derivatives, these fully-reduced arseno compounds are in-

effective against trypanosomes or spirochætes *in vitro*. More recent developments of this aspect of chemotherapeutics have resulted in the preparation of neosalvarsan, in which one substituted amino grouping, —NH . CH_2O . SONa, is present, and of sulpharsenol, in which two —NH . CH_2O . SO_2Na groupings are present. These have the advantage over salvarsan of being more convenient in administration, since they are less toxic in their immediate effects and have the further apparent advantage of being soluble in water at neutral reaction. But, despite this apparent advantage, equally good results seem to have been obtained with salvarsan, which is insoluble at the hydrogen ion concentration of the blood, as with these soluble derivatives. Silver salvarsan represents an example of a combination of a loose chemical nature between salvarsan and silver which has been extensively used in treating spirochætal infections, notably in Germany.

A recent development of a highly promising nature is the production by Fourneau and his collaborators (1923) of stovarsol, *p*-hydroxy-*m*-acetylaminophenylarsinic acid—



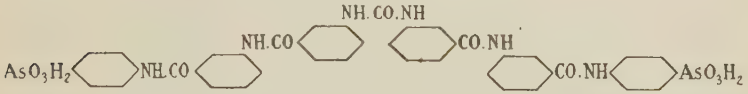
STOVARSOL

which, it is claimed, is effective in curing syphilis when administered by mouth, thus representing the first compound for which such a claim has been made. Stovarsol has recently been applied, again orally, to the treatment of amœbic dysentery. It appears to have a specific action on the intestinal lesions, and highly promising results, which have been confirmed by other investigators, are recorded.

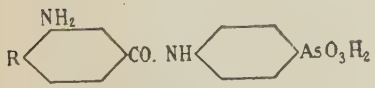
The non-acetylated compound treparsol (*p*-hydroxy-*m*-aminophenylarsinic acid) has likewise been used by

Flandin for the treatment of amœbiasis in conjunction with emetine given intravenously.

In an interesting study of the relation of trypanocidal action to chemical constitution recently commenced by King, an attempt has been made to combine the well-recognised trypanocidal action of compounds of the phenylarsinic acid series, to which tryparsamide belongs, with that of compounds related to Bayer 205 or Fourneau's 309. The intention was to attach two arsinic acid residues to the ends of a chain similar to that which links the naphthylamine sulphonic acid residues in 309. King and Murch have, indeed, synthesised compounds of the type—



and note that, although some of the simpler intermediate compounds showed trypanocidal activity, they themselves are totally devoid of any action on the trypanosomes, a striking illustration of the imperfection of our present theories in chemotherapy. It should, however, be remembered, as King points out, that such compounds are colloids of considerable molecular weight (936) and are but slightly soluble. This last property is not necessarily incompatible with therapeutical activity, as we have seen in the case of salvarsan, but nevertheless it is noteworthy that Bayer 205 (or Fourneau's 309), with its six sulphonic acid groups, is very soluble in water. In continuation of these investigations, King and Murch have prepared a number of derivatives of 3′-aminobenzoyl-4-aminophenylarsinic acid (Formula I), and find that when R = OCH_3 of Cl in the formula below



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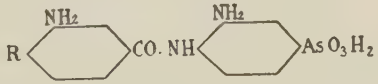
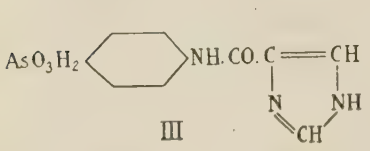


II

the resulting compound effected permanent cures in mice infected with *Trypanosoma equiperdum*. The 3′ : 3-diamino acids were also permanently curative in the instances where R = H, CH_3, or Cl (Formula II). Balaban and King have prepared similar derivatives of phenylarsinic acid in which a glyoxaline residue is present, and they find that glyoxaline-4′-carboxy-*p*-aminophenylarsinic acid (Formula III) and glyoxaline-4′-carboxy-*p*-amino-3-aminophenylarsinic acid (Formula IV) can both effect permanent cures.



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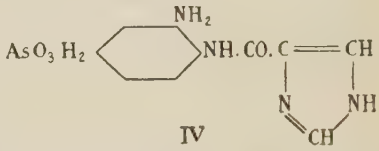
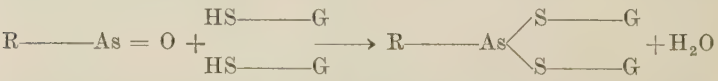


IV

The further outcome of these investigations, which are adding materially to our information concerning molecular configuration and trypanocidal activity, will be followed with interest.

With reference to the varying toxicities shown by organic arsenicals in which the arsenic is in different states of oxidation, mention may here be made of an interesting theory put forward by Voegtlin to explain the mechanism of the toxic action of these arseno compounds on living matter. He argues that the first step is an oxidation to the corresponding arsenoxide, which, as Ehrlich showed, is much the most directly toxic of the arsenicals. The arsenoxide then reacts with reduced glutathione of the tissues :—



The evidence on which this reaction is based is : (1) the death of animals given a fatal intravenous injection of an arsenoxide, such as 3-amino-4-hydroxyphenylarsenious oxide, is greatly delayed by a simultaneous injection of sodium thioglycollate or other sulphydryl compound ; (2) the feeding with moderately large amounts of the two constituent amino

acids of glutathione—glutaminic acid and cysteine—offers considerable protection against a lethal dose of an arsenoxide given two to three hours later. The inference is that the arsenoxide may be considered as a specific poison for reduced glutathione, and a toxic dose will therefore lead to cellular asphyxia. Parallel results have been obtained in investigations on infected rats. Various sulphydryl compounds (reduced glutathione, thioglycollic, thiolactic, thiosalicylic

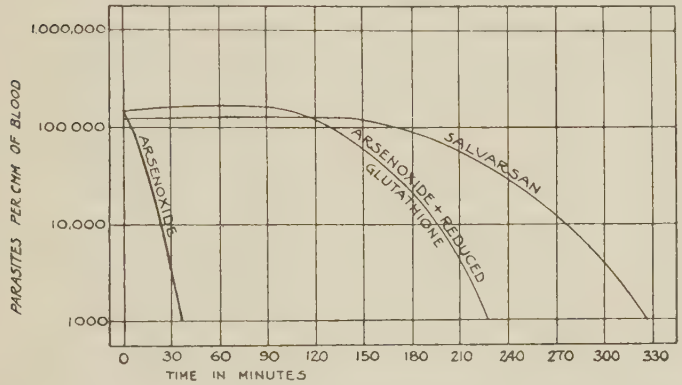


FIG. 38.—Trypanocidal action of salvarsan, arsenoxide plus reduced glutathione, and arsenoxide alone, in rats. (After Voegtlin.)

acids, cysteine, and glycylcysteine), if injected into the infected rat just before a minimum effective dose of an arsenoxide, greatly lower the rate of disappearance of the trypanosomes from the blood. Indeed, the arsenoxide, instead of proving immediately lethal, as it is when injected alone, shows a latent period very similar to that which salvarsan itself exhibits (see Fig. 38). From the positive nitroprusside test given by trypanosomes Voegtlin assumes the presence of sulphydryl compounds in these parasites, and postulates a mechanism essentially similar to the one responsible for the toxic action of arsenoxide on mammalian tissue. Such views are highly interesting and suggestive,

but they still do not explain the selective action of the arsenicals on the parasites. This, however, might be a question of " sulphydryl reserve," or, as Voegtlin suggests, might depend on the tissues having a higher oxidation potential for arsenoxide than the parasites, whereby a more rapid conversion to the non-atoxic pentavalent arsenic compounds would result, and elimination take place before appreciable cellular asphyxia could occur in the tissue cells of the host.

It is a natural step from arsenic derivatives to investigate the therapeutic action of compounds of the closely-related metals antimony and bismuth. The type of compound which has been used is much simpler than the complex arsenicals which we have been considering. Tartar emetic and its sodium analogue and the corresponding bismuthyl tartrates have been used, both *in vitro* and in clinical practice, against trypanosomes and spirochætes. Here it is found that the results obtained *in vitro* and *in vivo* are parallel, and the action of these compounds seems to consist in a direct toxic effect on the parasites which is more or less proportional to the amount of metal—antimony or bismuth—present. Indeed, metallic antimony suspended in oil and injected intramuscularly is as effective as tartar emetic in combating trypanosomiasis, Whilst antimony is more toxic than bismuth for trypanosomes, the reverse relationship holds in the case of spirochætes. Mercury in various forms has been used for many years as a specific in cases of syphilis, and it is natural that considerable attention has been given to the elaboration of complex organic mercurials. But here, again, the toxicity to parasites seems to be proportional to the amount of mercury present, since all attempts to alter the C/T ratio have failed. The inference is that in these instances also one is dealing with a direct lethal action on the parasite. The continued activity in the elaboration of new mercury compounds would seem to be a response to the clinicians' demand in countering syphilis for a short intensive

arsenic treatment together with a prolonged mercury treatment.

(4) **Alkaloids.**—The treatment of malarial infections with the alkaloids of cinchona bark, more especially quinine, is a very old therapeutic measure, but, despite the preparation within recent years of many artificial derivatives of these alkaloids, none shows any real advantage over quinine. With our improving knowledge of the alkaloids occurring in this bark—and it is estimated that there are more than twenty—it has been found that of these alkaloids hydroquinine, quinidine, and cinchonidine are at least no less toxic to the parasite, and not any more toxic to man, than is quinine. This is of interest, as Dale points out, in demonstrating that the toxicity of quinine to the malarial protozöon is not dependent on the vinyl group (see the formula on p. 366), nor on the methoxy group, nor on the stereo configuration of this alkaloid, since the first-mentioned group is reduced to ethyl in hydroquinine, the methoxy group is absent in cinchonidine, and quinidine differs from quinine in at least one of its asymmetric carbon atoms. Here, again, it is to be noted that quinine, although toxic *in vivo*, is devoid of parasiticidal action *in vitro*.

As in the case of malaria, so tropical (amœbic) dysentery has been combated by the use of alkaloids since at least the seventeenth century. Of the alkaloids of ipecacuanha root, the specific for dysentery introduced by Le Gras in 1672, cephæline, $C_{28}H_{38}O_4N_2$, and its methyl ester emetine, $C_{29}H_{40}O_4N_2$, have been identified, and both possess atoxic action on *Entamœba histolytica* much more marked than that of any artificial derivatives prepared from them. Earlier experiments carried out *in vitro* on the action of emetine on *E. histolytica* were interpreted as showing a simple direct toxic action of the alkaloid on the parasite, but more recent investigations by Dale and Dobell (1917) and Allen (1920) have shown that emetine is very much less toxic *in vitro* than was at first stated, and, indeed, so far as *in vitro* experi-

ments go, quinine is much more effective than emetine, although the former is quite devoid of any effect *in vivo*, and consequently of no curative value in this disease. Again, Dale and Dobell found one of the less important alkaloids of ipecacuanha root, namely, methyl psychotrine, to be strongly toxic to the isolated amœbæ, and almost non-toxic to them in the mammal. These illustrations demonstrate the extreme complexity of what was at first an apparently simple question in chemotherapeutics, and again emphasise the complete failure of results obtained *in vitro* to correlate with those obtained *in vivo*.

Chemotherapy of Bacterial Diseases

In the search for chemical substances specifically toxic to pathogenic bacteria much less striking results are to be recorded than in the case of protozöal diseases. Investiga-

TABLE XXII

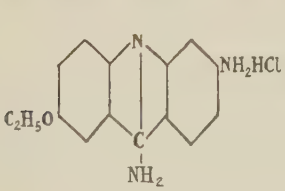
Comparative Action of Antiseptics in Water and in Blood Serum

Substance.	*Staphylococcus aureus.*		*Bacillus coli comm.*	
	(*a*)	(*b*)	(*a*)	(*b*)
Mercuric chloride .	1 : 1,000,000	1 : 10,000	1 : 1,000,000	1 : 10,000
Phenol . . .	1 : 250	1 : 250	1 : 500	1 : 500
Malachite green .	1 : 10,000,000	1 : 40,000	1 : 20,000	1 : 1,000
Brilliant green .	1 : 10,000,000	1 : 30,000	1 : 130,000	1 : 3,500
Crystal violet .	1 : 4,000,000	1 : 400,000	1 : 8,000	1 : 8,000
Trypaflavine . .	1 : 20,000	1 : 200,000	1 : 1,300	1 : 100,000

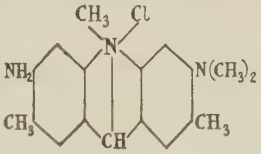
tions in this field have frequently ended in the discovery that an antiseptic powerfully bactericidal externally proved quite innocent *in vivo*, the most probable cause for such discrepancies being the presence of blood proteins, which were

demonstrated by Bechhold in many cases to lower the toxicity of an antiseptic even *in vitro* by forming a direct combination with the latter. This effect is illustrated in the table shown on p. 360 (Heffter, vol. i., 1259), which gives the minimum lethal concentrations of the various antiseptics, (*a*) in water and (*b*) in blood serum.

From time to time numerous dyestuffs with specific staining reactions for given bacteria have been investigated with the view of utilisation as bactericides *in vivo*, but many of these are found to inhibit the phagocytic action of leucocytes, *e.g.*, on *Staphylococcus*, in dilutions below that at which their bactericidal action ceases, and thus, in addition to the inhibiting action of the blood proteins on the antiseptics, we have to reckon with an inhibiting action of the antiseptic on one of the natural defence mechanisms of the body. More successful results have been recorded in the cases of



RIVANOL



FLAVICIDE

p-methoxy-Malachite green and Ethyl violet of the triphenylmethane series, and notably in the cases of Proflavine and Trypaflavine (Acriflavine), of the acridine series of dyestuffs. These latter, which are more powerfully bactericidal in the presence than in the absence of serum proteins (see Table XXII), have already been mentioned as having some application in trypanosomiasis (p. 349). More recently Morgenroth (1921) and his collaborators have introduced rivanol, which is the hydrochloride of 2-ethoxy-6 : 9-diaminoacridine, and Langer (1922) has utilised flavicide, 2 : 7-dimethyl - 6 - dimethylamino - 3 - amino - 10 -methylacridinium chloride ; but, promising as these substances are, there is no

conclusive evidence as yet to show that they are capable of curing bacterial septicæmias. This, however, has been more nearly attained by the use of an artificial derivative of the cinchona series of alkaloids, namely, ethylhydrocupreine (optochin), first used by Morgenroth and his collaborators in 1911–12.

Optochin is toxic to several strains of pneumococcus both *in vitro* and *in vivo*, and in conjunction with specific anti-pneumococcal sera gives results much greater than would be obtained by summation of these two individual effects. From the results obtained with optochin and many other artificial cinchona alkaloids it would appear that to obtain the optimal bactericidal effect *in vivo* the co-operation of the host's defensive mechanisms is necessary, and that these latter are readily impaired or suppressed by over-administration of the alkaloids, even though the amount injected is well within the host's tolerance (Felton and Dougherty, 1922).

Chemotherapy of Tuberculosis.—Tuberculosis is a disease which has proved to be even more resistant to chemotherapeutical treatment than other bacterial or bacillary infections, despite numerous attempts to utilise remedies which gave promising results in other cases. This resistance may be ascribed to the physical nature of the bacillus, which possesses a fatty envelope probably impermeable to most of the substances used, or to the very poor blood supply which characterises tubercular lesions, rendering uncertain the access of the curative agent to the site of the infection. Nevertheless, from time to time partially successful results have been claimed, more especially on behalf of metallic derivatives, amongst them those of gold. None of the earlier efforts seem to have stood the test of time, but recently Møllgaard, a Danish physician, has introduced a gold salt, sodium aurithiosulphate, of the composition—

$$[S_2O_3Au]\,{}^{Na}_{Na_2S_2O_3},$$

which he has registered under the name of Sanocrysin, and in which the gold is present in a non-ionisable form. This salt is now receiving a great deal of attention as a specific curative agent in tuberculosis on account of the highly promising preliminary results obtained by Møllgaard and his associates. It undoubtedly has a specific action on tissues infected by tubercle bacilli, but it remains to be seen whether or not this action can be made the basis of a cure. On injection into tuberculous patients it produces a typical reaction with rise in temperature, albuminuria, etc., which is not shown by the normal subject, and the severity of which is more or less proportional to the extent of the infection. The treatment may be combined with a specific serum treatment aimed at combating the toxic reaction. The most promising results are those obtained in mild infections; advanced pulmonary cases are much less tractable.

External Antiseptics

In the field of external antiseptics it has not been difficult to find numerous exceedingly effective chemical compounds having many useful applications *in vitro*, and also for the local external treatment of infected wounds and mucous surfaces.

In selecting an antiseptic for any given purpose several points must be considered, as, for example, the following :—

(1) The nature of the medium in which the antiseptic is to act is of great importance. Many antiseptics have a marked affinity for proteins and fats, with which they may form more or less stable compounds which are in general much less reactive than the parent substance. The exceptional case of the acridine dyestuffs may again be mentioned here; these, as Browning has shown, are more reactive in the presence of proteins than in their absence.

(2) The nature of the particular organism to be dealt with must be considered. It may have a waxy or chitinous envelope which resists the entrance of a given antiseptic, or,

again, the spores may be much more resistant than the parent organism.

(3) Many antiseptics show a highly specific action, being toxic to one type of organism and non-toxic to another which may be closely related to the first. This specificity of action is utilised in bacteriology for the selection of one species of bacteria out of a mixture of many, an antiseptic being chosen which inhibits the growth of the other organisms more than that of the one which it is desired to select.

(4) The capacity of many organisms to develop a specific resistance on prolonged treatment with a given antiseptic has already been mentioned, and has an obvious importance here. Most biochemists cannot fail to have observed the growth of moulds, etc., in solutions which might be expected to be quite toxic, *e.g.*, in 10 per cent. sulphuric acid or sodium hydroxide, an illustration of the capacity of lowly organisms to adapt themselves to exceedingly unpromising environments.

(5) An antiseptic may inhibit the growth or reproduction of bacteria, or in other ways interfere with their metabolism, without killing them. On removal of the antiseptic they may again flourish.

Bearing in mind these observations, it is not surprising that the ideal antiseptic still awaits discovery, and the task is obviously difficult, since a substance toxic to all micro-organisms will probably be toxic to other living cells. External antiseptics are of many types—halogen, sulphur, bismuth, silver, and mercury compounds, phenol and formaldehyde derivatives, dyestuffs and alkaloids —but these cannot be considered in detail here, many having attained but a fleeting popularity. Nevertheless recent progress with antiseptics of the type of chloramine T, the acridine dyestuffs, and the artificial quinine alkaloids is highly promising.

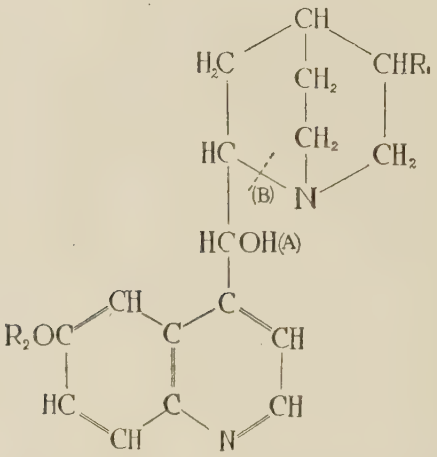
Chlorine, in the form of hypochlorite solutions, has a wide application as an antiseptic, but most hypochlorites are too unstable in dilute aqueous solutions to be really serviceable.

Dakin, and later Carrel, found that the addition of boric acid greatly increased the stability of hypochlorite solutions, and such combinations were extensively employed during the war of 1914–18. Dakin continued his search for still more effective chlorine compounds which would liberate this element in contact with a wound or sore, and as a result he has produced chloramine T and dichloramine T, the most notable chlorine antiseptics yet discovered. Chloramine T is a soluble sodium derivative of *p*-toluenesulphonchloramide, $CH_3 . C_6H_4 . SO_2Na : NCl$, whilst dichloramine T is an insoluble modification containing two chlorine atoms, $CH_3 . C_6H_4 . SO_2Cl : NCl$ or $CH_3 . C_6H_4 . SO_2 . NCl_2$. These antiseptics are stable in the dark, are free from irritant properties, and are very useful in a great variety of septic conditions.

Of the dyestuffs many have been used, and suitability is obviously determined by particular cases. The triphenylmethanes and the acridines provide the most useful external antiseptics. It is interesting to note that one of the first dyestuffs to reveal its antiseptic properties—Malachite green —still remained in use during the late war. The acridines, already considered as internal antiseptics, are now recognised as the most potent of external dyestuff antiseptics, and seem destined to retain that position for some time, whereas they are likely to be superseded as internal antiseptics.

The efficiency of quinine as an internal antiseptic in malaria has already been noted, and we have seen that of the numerous artificial derivatives of the cinchona alkaloids none has proved more efficacious against the parasite than the natural compounds. Nevertheless many of these artificial alkaloids have useful applications as external (and sometimes as internal, *e.g.*, optochin and vuzin) antiseptics in other directions. We owe an interesting series of investigations on the potency of these substituted hydrocupreine derivatives principally to Morgenroth and his collaborators, from which same correlation between constitution and antiseptic action

emerges. The relationship of these antiseptics will be seen from the formula :—



In quinine R_1 is —CH = CH_2, the vinyl radicle, whilst in hydrocupreine this is reduced to —CH_2—CH_3, ethyl. In quinine R_2 is methyl, and it is this substituent which has been varied in the series which we are considering. Some of the results obtained are summarised in the table given (p. 367). It is clear that the antiseptic action *in vitro* of these derivatives is maximal against diphtheria, tetanus, anthrax and gas gangrene bacilli, and against streptococcus, when R_2 is the iso-octyl radicle, the toxicity gradually increasing as the substituent becomes larger until this eight-membered carbon chain is reached, and then diminishing again, with a further continued increase in the complexity of the side chain. Against other bacilli similar results, generally showing a different optimal size of side chain, are noted. Thus for *Staphylococcus aureus* the heptyl radicle is most deadly, and for pneumococcus and trypanosomes the ethyl radicle, as in optochin. With regard to the relationship of other parts of the quinine structure to toxicity, the fact that reduction of

Table XXIII

Limiting Dilutions of Toxic Action of Artificial Quinine Derivatives on Bacilli

Quinine Derivative.	Diphtheria Bacillus.		Lethal Concentration for Bacilli.			
	Antiseptic Action.	Lethal in presence of Proteins.	Tetanus.	Anthrax.	Gas Gangrene.	Strepto-coccus.
1. Quinine hydrochloride (methyl cupreine).	1 : 10,000	1 : 1,000	1 : 1,000	1 : 500	—	1 : 1,000
2. Hydrocupreine diHCl . . .	1 : 10,000	—	—	—	—	—
3. Hydroquinine HCl (methyl hydrocupreine).	1 : 10,000	—	—	—	—	1 : 1,000
4. Optochin HCl (ethyl hydrocupreine) .	1 : 10,000	1 : 5,000	1 : 2,500	1 : 2,000	1 : 2,500	—
5. *Iso*-propyl hydrocupreine HCl . .	1 : 12,500	—	—	—	—	—
6. *Iso*-propyl hydrocupreine diHCl .	1 : 25,000	—	—	—	—	1 : 8,000
7. *Iso*-butyl hydrocupreine HCl . .	1 : 50,000	—	—	—	—	—
8. *Iso*-butyl hydrocupreine diHCl .	1 : 62,500	—	—	—	—	1 : 16,000
9. Eucupin (*iso*-amyl hydrocupreine diHCl).	1 : 200,000	1 : 60,000	1 : 20,000	1 : 30,000	1 : 25,000	1 : 40,000
10. Hexyl hydrocupreine diHCl . .	1 : 300,000	—	—	—	—	—
11. Heptyl hydrocupreine diHCl . .	1 : 500,000	—	—	—	—	—
12. Vuzin (*iso*-octyl hydrocupreine diHCl)	1 : 750,000	1 : 60,000	1 : 60,000	1 : 60,000	1 : 50,000	1 : 80,000
13. Decyl hydrocupreine diHCl . .	1 : 500,000	1 : 10,000	1 : 10,000	1 : 10,000	1 : 25,000	1 : 20,000
14. Dodecyl hydrocupreine diHCl . .	1 : 100,000	1 : 10,000	1 : 7,500	1 : 6,000	—	1 : 10,000
15. Cetyl hydrocupreine diHCl . .	1 : 5,000	—	—	—	—	—

the vinyl group to ethyl (quinine ⟶ hydroquinine) does not alter the therapeutic action in malaria has already been mentioned, and this point is further demonstrated in the table. Nevertheless, complete oxidation of the side chain to carboxyl destroys all toxicity, as in quitenine. Other observations bearing on this question are that alteration of the secondary alcohol group at (A) does not affect the toxicity of quinine derivatives, nor does an opening of one of the rings at (B), as in quinatoxine, destroy it, but it is interesting to note that trypanosomes resistant to the original quinine alkaloid no longer display this resistance to the corresponding quinatoxine, whilst those resistant to the toxine are likewise resistant to the quinine. Morgenroth assumes that quinatoxines have a greater affinity for the " quinine receptor " of the parasite, or host's tissues, than has quinine itself, and so correlates these results, the inference then being that integrity of the ring at the point (B) is not an essential factor.

It is clear from the foregoing survey of the chemotherapy of protozöal and bacterial infections that no one theory, simple or otherwise, will accommodate all the known facts. We have, in the first place, the direct toxic action of mercury, antimony, and bismuth derivatives, both *in vitro* and *in vivo*, which apparently depends simply on the introduction of a highly toxic metal in some suitable physical state into the organism. Secondly, we have many cases, including all the really effective agents, in which the toxicity *in vitro* bears little relationship to that shown in the living animal. Thus the toxicity of quinine to the malarial and dysenteric parasites is summarised in the following table :—

	In vitro.	*In vivo.*
Parasiticidal effect of quinine on :—		
Malarial *Plasmodium*	—	+
Entamœba histolytica	+	—

Again, we have the great lowering in the toxicity of Malachite green in the presence of serum, and the corresponding increase in that of Trypaflavine under the same conditions,

and the powerfully toxic action of salvarsan and Bayer 205, which is only revealed *in vivo*. Thirdly, a point of great importance is the marked specificity of many of these antiseptics (see Tables XXII and XXIII), a specificity which sometimes differentiates in a remarkable manner between two closely-related organisms. And lastly may be mentioned the claims which have been made on behalf of Bayer 205 in relation to conferring on the host an immunity to the infective parasites in addition to destroying those actually present in the tissues. All these facts are exceedingly difficult to reconcile with Ehrlich's " chemoreceptor " theory, faithful servant as it has proved itself ; nor, indeed, does any other single theory since propounded offer a better explanation. In particular cases, *e.g.*, the arsenicals, more detailed experimental investigation is beginning to hint at the wider vision, and, indeed, it is clear that in all cases the substance non-toxic *in vitro* must undergo alteration in contact with the host's tissues, or induce some chemical or physical change in the tissues leading to production of the toxic substance or condition. The complexity of the whole question is still further increased by a factor which has not previously been discussed here, namely, the development of strains of parasites which, after being subjected to the action of the antiseptic, acquire a resistance towards it. It is possible to produce such resistant strains *in vitro* without the co-operation of the host's tissues ; thus pneumococci have been developed to resist the action of optochin, and certain cholera bacilli have likewise been modified in serial media containing increasing amounts of the toxic substance to resist both dyestuffs and arsenic derivatives. It is interesting to note that in the latter case the resistance to antimony was found to have risen too ; and, indeed, Gonder (1912) has recorded that resistance to tartar emetic in the case of protozöa is better attained by treatment with arsenious acid than with an antimony compound ! Such resistant strains may also develop *in vivo*, and the resistance is generally found to be

markedly specific ; thus a strain of trypanosomes resistant to Trypan red might still retain its full sensitivity to atoxyl, and two or more resistances may be developed in the same strain independently of one another. One of the most interesting observations recorded in this field is that in which a trypanosome strain, resistant to atoxyl in the mouse, lost this and became normally sensitive in the rat during the course of forty passages through the second host, and immediately regained its original resistance on being retransferred to its first host (Mesnil and Brimont, 1908). Of a similar nature is an observation that a strain of dysentery amœbæ, readily susceptible to emetine in the human, became quite resistant when transferred to the kitten. In these cases, as Dale expresses it, " to suggest, as an explanation, co-operation of the host's tissues, in a manner concerning which the knowledge yet available affords not even a hint, is unsatisfactory ; but it seems at present the only alternative to a completely agnostic attitude." Although the chemotherapy of infectious diseases has not yet realised Ehrlich's ideal—*therapia sterilisans magna*—in which a single large injection would effect a complete cure, there is no reason to doubt that more and more potent remedies will be forthcoming as our information of the physical, chemical, and physiological reactions of the internal antiseptics increases, and meanwhile the successes already achieved in what were previously regarded as practically intractable diseases, partial though many of these successes are, constitute noteworthy advances.

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The author acknowledges his indebtedness for much of the material embodied in this chapter to an article by Dr. H. H. Dale on " Chemotherapy " (*Physiol. Reviews*, 1923, **3**, 359). The reader may consult the following references in addition to those which will be found in the above-mentioned article.

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